



## INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

<b>(51) International Patent Classification <sup>7</sup> :</b> <b>C12Q 1/68</b>	<b>A1</b>	<b>(11) International Publication Number:</b> <b>WO 00/42219</b> <b>(43) International Publication Date:</b> 20 July 2000 (20.07.00)
<b>(21) International Application Number:</b> PCT/US00/00388 <b>(22) International Filing Date:</b> 6 January 2000 (06.01.00) <b>(30) Priority Data:</b> 09/229,007 12 January 1999 (12.01.99) US <b>(63) Related by Continuation (CON) or Continuation-in-Part (CIP) to Earlier Application</b> US 09/229,007 (CON) Filed on 12 January 1999 (12.01.99) <b>(71) Applicant (for all designated States except US):</b> SANGAMO BIOSCIENCES, INC. [US/US]; Point Richmond Tech Center, Suite A100, 501 Canal Boulevard, Richmond, CA 94804 (US). <b>(72) Inventors; and</b> <b>(75) Inventors/Applicants (for US only):</b> EISENBERG, Stephen, P. [US/US]; 5664 Pennsylvania Place, Boulder, CO 80303 (US). CASE, Casey, C. [US/US]; 605 Barroilhet Avenue, San Mateo, CA 94402 (US). COX, George, N., III [US/US]; 678 West Willow Street, Louisville, CO 80027 (US). JAMIESON, Andrew [GB/US]; 2528 Sutter Street, San		Francisco, CA 94115 (US). REBAR, Edward, J. [US/US]; 815 Regal Road, Berkeley, CA 94708 (US). <b>(74) Agents:</b> LIEBESCHUETZ, Joe et al.; Townsend and Townsend and Crew LLP, Two Embarcadero Center, 8th floor, San Francisco, CA 94111-3834 (US). <b>(81) Designated States:</b> AE, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CR, CU, CZ, DE, DK, DM, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG). <b>Published</b> <i>With international search report.</i>
<b>(54) Title:</b> SELECTION OF SITES FOR TARGETING BY ZINC FINGER PROTEINS AND METHODS OF DESIGNING ZINC FINGER PROTEINS TO BIND TO PRESELECTED SITES		
<b>(57) Abstract</b> <p>The invention provides criteria and methods for selecting optimum subsequence(s) from a target for targeting by a zinc finger protein. Some of the methods of targetgene site selection seek to identify one or more target segments having a DNA motif containing one or more so-called D-able subsites having the sequence 5'NNGK3'. Other methods of the invention are directed to selection of target segments within target genes using a correspondence regime between different triplets of three bases and the three possible positions of a triplet within a nine-base site. In another aspect, the invention provides methods of designing zinc finger proteins that bind to a preselected target site. These methods can be used following the preselection of target sites according to the procedures and criteria described above. The methods of design use a database containing information about previously characterized zinc finger proteins.</p>		

*FOR THE PURPOSES OF INFORMATION ONLY*

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AL	Albania	ES	Spain	LS	Lesotho	SI	Slovenia
AM	Armenia	FI	Finland	LT	Lithuania	SK	Slovakia
AT	Austria	FR	France	LU	Luxembourg	SN	Senegal
AU	Australia	GA	Gabon	LV	Latvia	SZ	Swaziland
AZ	Azerbaijan	GB	United Kingdom	MC	Monaco	TD	Chad
BA	Bosnia and Herzegovina	GE	Georgia	MD	Republic of Moldova	TG	Togo
BB	Barbados	GH	Ghana	MG	Madagascar	TJ	Tajikistan
BE	Belgium	GN	Guinea	MK	The former Yugoslav Republic of Macedonia	TM	Turkmenistan
BF	Burkina Faso	GR	Greece	ML	Mali	TR	Turkey
BG	Bulgaria	HU	Hungary	MN	Mongolia	TT	Trinidad and Tobago
RJ	Benin	IE	Ireland	MR	Mauritania	UA	Ukraine
BR	Brazil	IL	Israel	MW	Malawi	UG	Uganda
BY	Belarus	IS	Iceland	MX	Mexico	US	United States of America
CA	Canada	IT	Italy	NE	Niger	UZ	Uzbekistan
CF	Central African Republic	JP	Japan	NL	Netherlands	VN	Viet Nam
CG	Congo	KE	Kenya	NO	Norway	YU	Yugoslavia
CH	Switzerland	KG	Kyrgyzstan	NZ	New Zealand	ZW	Zimbabwe
CI	Côte d'Ivoire	KP	Democratic People's Republic of Korea	PL	Poland		
CM	Cameroon	KR	Republic of Korea	PT	Portugal		
CN	China	KZ	Kazakhstan	RO	Romania		
CU	Cuba	LC	Saint Lucia	RU	Russian Federation		
CZ	Czech Republic	LI	Licchtenstein	SD	Sudan		
DE	Germany	LK	Sri Lanka	SE	Sweden		
DK	Denmark	LR	Liberia	SG	Singapore		
EE	Estonia						

5                   SELECTION OF SITES FOR TARGETING BY ZINC FINGER PROTEINS AND  
                  METHODS OF DESIGNING ZINC FINGER PROTEINS TO BINDS TO  
                  PRESELECTED SITES

TECHNICAL FIELD

10               The invention resides in the technical fields of bioinformatics, and protein  
                  engineering.

BACKGROUND

                  Zinc finger proteins (ZFPs) are proteins that can bind to DNA in a  
                  sequence-specific manner. Zinc fingers were first identified in the transcription factor  
15   TFIIIA from the oocytes of the African clawed toad, *Xenopus laevis*. An exemplary  
                  motif characterizing one class of these protein (C<sub>2</sub>H<sub>2</sub> class) is -Cys-(X)<sub>2-4</sub>-Cys-(X)<sub>12</sub>-His-  
                  (X)<sub>3-5</sub>-His (where X is any amino acid). A single finger domain is about 30 amino acids  
                  in length, and several structural studies have demonstrated that it contains an alpha helix  
                  containing the two invariant histidine residues and two invariant cysteine residues in a  
20   beta turn co-ordinated through zinc. To date, over 10,000 zinc finger sequences have been  
                  identified in several thousand known or putative transcription factors. Zinc finger  
                  domains are involved not only in DNA-recognition, but also in RNA binding and in  
                  protein-protein binding. Current estimates are that this class of molecules will constitute  
                  about 2% of all human genes.

25               The x-ray crystal structure of Zif268, a three-finger domain from a murine  
                  transcription factor, has been solved in complex with a cognate DNA-sequence and  
                  shows that each finger can be superimposed on the next by a periodic rotation. The  
                  structure suggests that each finger interacts independently with DNA over 3 base-pair  
                  intervals, with side-chains at positions -1, 2, 3 and 6 on each recognition helix making  
30   contacts with their respective DNA triplet subsites. The amino terminus of Zif268 is  
                  situated at the 3' end of the DNA strand with which it makes most contacts. Recent  
                  results have indicated that some zinc fingers can bind to a fourth base in a target segment.

If the strand with which a zinc finger protein makes most contacts is designated the target strand, some zinc finger proteins bind to a three base triplet in the target strand and a fourth base on the nontarget strand. The fourth base is complementary to the base immediately 3' of the three base subsite.

5                   The structure of the Zif268-DNA complex also suggested that the DNA sequence specificity of a zinc finger protein might be altered by making amino acid substitutions at the four helix positions (-1, 2, 3 and 6) on each of the zinc finger recognition helices. Phage display experiments using zinc finger combinatorial libraries to test this observation were published in a series of papers in 1994 (Rebar et al., *Science* 10   263, 671-673 (1994); Jamieson et al., *Biochemistry* 33, 5689-5695 (1994); Choo et al, *PNAS* 91, 11163-11167 (1994)). Combinatorial libraries were constructed with randomized side-chains in either the first or middle finger of Zif268 and then used to select for an altered Zif268 binding site in which the appropriate DNA sub-site was replaced by an altered DNA triplet. Further, correlation between the nature of introduced 15   mutations and the resulting alteration in binding specificity gave rise to a partial set of substitution rules for design of ZFPs with altered binding specificity.

                  Greisman & Pabo, *Science* 275, 657-661 (1997) discuss an elaboration of the phage display method in which each finger of a Zif268 was successively randomized and selected for binding to a new triplet sequence. This paper reported selection of ZFPs 20   for a nuclear hormone response element, a p53 target site and a TATA box sequence.

                  A number of papers have reported attempts to produce ZFPs to modulate particular target sites. For example, Choo et al., *Nature* 372, 645 (1994), report an attempt to design a ZFP that would repress expression of a bcr-abl oncogene. The target segment to which the ZFPs would bind was a nine base sequence 5'GCA GAA3' GCC 25   chosen to overlap the junction created by a specific oncogenic translocation fusing the genes encoding bcr and abl. The intention was that a ZFP specific to this target site would bind to the oncogene without binding to abl or bcr component genes. The authors used phage display to screen a mini-library of variant ZFPs for binding to this target segment. A variant ZFP thus isolated was then reported to repress expression of a stably 30   transfected bcr-abl construct in a cell line.

                  Pomerantz et al., *Science* 267, 93-96 (1995) reported an attempt to design a novel DNA binding protein by fusing two fingers from Zif268 with a homeodomain from Oct-1. The hybrid protein was then fused with a transcriptional activator for

expression as a chimeric protein. The chimeric protein was reported to bind a target site representing a hybrid of the subsites of its two components. The authors then constructed a reporter vector containing a luciferase gene operably linked to a promoter and a hybrid site for the chimeric DNA binding protein in proximity to the promoter. The authors  
5 reported that their chimeric DNA binding protein could activate expression of the luciferase gene.

Liu et al., *PNAS* 94, 5525-5530 (1997) report forming a composite zinc finger protein by using a peptide spacer to link two component zinc finger proteins each having three fingers. The composite protein was then further linked to transcriptional  
10 activation domain. It was reported that the resulting chimeric protein bound to a target site formed from the target segments bound by the two component zinc finger proteins. It was further reported that the chimeric zinc finger protein could activate transcription of a reporter gene when its target site was inserted into a reporter plasmid in proximity to a promoter operably linked to the reporter.

15 Choo et al., WO 98/53058, WO98/53059, and WO 98/53060 (1998) discuss selection of zinc finger proteins to bind to a target site within the HIV Tat gene. Choo et al. also discuss selection of a zinc finger protein to bind to a target site encompassing a site of a common mutation in the oncogene ras. The target site within ras was thus constrained by the position of the mutation.

20 None of the above studies provided criteria for systematically evaluating the respective merits of the different potential target sites within a candidate gene. The phage display studies by Rebar et al., supra, Jamieson et al., supra and Choo et al., *PNAS*, (1994) supra, all focused on alterations of the natural Zif268 binding-site, 5'GCG TGG GCGc3', and were not made with reference to a predetermined target gene. Choo et  
25 al. *Nature* (1994), supra's selection of target site was constrained solely by the intent that the site overlap the interface between bcr and abl segments and did not involve a comparison of different potential target sites. Likewise, Greisman & Pabo chose certain target sites because of their known regulatory roles and did not consider the relative merits of different potential target segments within a preselected target gene. Similarly,  
30 Choo et al. (1998), supra's choice of target site within ras was constrained by the position of a mutation. No criterion is provided for Choo et al. (1998)'s selection of a target site in HIV Tat. Finally, both Pomerantz et al., supra and Liu et al., supra constructed artificial

hybrid target sites for composite zinc fingers and then inserted the target sites into reporter constructs.

### SUMMARY OF THE INVENTION

5           The invention provides methods of selecting a target site within a target sequence for targeting by a zinc finger protein. Some such methods comprise providing a target nucleic acid to be targeted by a zinc finger protein and outputting a target site within the target nucleic acid comprising 5'NNx aNy bNzc3'. Each of (x, a), (y, b) and (z, c) is (N, N) or (G, K) provided at least one of (x, a), (y, b) and (z, c) is (G, K). N and  
10 K are IUPAC-IUB ambiguity codes. In some methods, a plurality of segments within the target nucleic acid are selected and a subset of the plurality of segments comprising 5'NNx aNy bNzc3' is output. Typically the target nucleic acid comprises a target gene. In some methods, at least two of (x, a), (y, b) and (z, c) is (G, K). In some methods, all three of (x, a), (y, b) and (z, c) are (G, K). Some methods further comprise identifying a  
15 second segment of the gene comprising 5'NNx aNy bNzc3', wherein each of (x, a), (y, b) and (z, c) is (N, N) or (G, K); at least one of (x, a), (y, b) and (z, c) is (G, K). and N and K are IUPAC-IUB ambiguity codes. In some methods, in the second segment at least two of (x, a), (y, b) and (z, c) are (G, K). In some methods, all three of at least one of (x, a), (y, b) and (z, c) are (G, K). In some methods, the first and second segments are separated  
20 by fewer than 5 bases in the target site.

          Some methods further comprise synthesizing a zinc finger protein comprising first, second and third fingers that bind to the bNz aNy and NNx triplets respectively. In some such methods, the synthesizing step comprises synthesizing a first zinc finger protein comprising three zinc fingers that respectively bind to the NNx aNy  
25 and bNz triplets in the target segment and a second three fingers that respectively bind to the NNx aNy and bNz triplets in the second target segment. In some methods, each of the first, second and third fingers is selected or designed independently. In some methods, a finger is designed from a database containing designations of zinc finger proteins, subdesignations of finger components, and nucleic acid sequences bound by the zinc  
30 finger proteins. In some methods, a finger is selected by screening variants of a zinc finger binding protein for specific binding to the target site to identify a variant that binds to the target site.

Some methods further comprise contacting a sample containing the target nucleic acid with the zinc finger protein, whereby the zinc finger protein binds to the target site revealing the presence of the target nucleic acid or a particular allelic form thereof. In some methods, a sample containing the target nucleic acid is contacted with  
5 the zinc finger protein, whereby the zinc finger protein binds to the target site thereby modulating expression of the target nucleic acid.

In some methods, the target site occurs in a coding region. In some methods, the target site occurs within or proximal to a promoter, enhancer, or transcription start site. In some methods, the target site occurs outside a promoter,  
10 regulatory sequence or polymorphic site within the target nucleic acid.

In another aspect, the invention provides alternate methods for selecting a target site within a polynucleotide for targeting by a zinc finger protein. These methods, comprising providing a polynucleotide sequence and selecting a potential target site within the polynucleotide sequence; the potential target site comprising contiguous first,  
15 second and third triplets of bases at first, second and third positions in the potential target site. A plurality of subscores are then determined by applying a correspondence regime between triplets and triplet position in a sequence of three contiguous triplets, wherein each triplet has first, second and third corresponding positions, and each combination of triplet and triplet position has a particular subscore. A score is then calculated for the  
20 potential target site by combining subscores for the first, second, and third triplets. The selecting, determining and calculating steps are then repeated at least once on a further potential target site comprising first, second and third triplets at first, second and third positions of the further potential target site to determine a further score. Output is then provided of at least one potential target site with its score. In some methods, output is  
25 provided of the potential target site with the highest score. In some methods, output is provided of the n potential target sites with the highest scores, and the method further comprises providing user input of a value for n. In some methods, the subscores are combined by forming the product of the subscores. In some methods, the correspondence regime comprises 64 triplets, each having first, second, and third corresponding positions,  
30 and 192 subscores.

In some methods, the subscores in the correspondence regime are determined by assigning a first value as the subscore of a subset of triplets and corresponding positions, for each of which there is an existing zinc finger protein that

comprising a finger that specifically binds to the triplet from the same position in the existing zinc finger protein as the corresponding position of the triplet in the correspondence regime; assigning a second value as the subscore of a subset of triplets and corresponding positions, for each of which there is an existing zinc finger protein that  
5 comprises a finger that specifically binds to the triplet from a different position in the existing zinc finger protein than the corresponding position of the triplet in the correspondence regime; and assigning a third value as the subscore of a subset of triplets and corresponding positions for which there is no existing zinc protein comprising a finger that specifically binds to the triplet.

10 In some methods, a context parameter with the subscore of at least one of the first, second and third triplets to give a scaled subscore of the at least one triplet. In some methods the context parameter is combined with the subscore when the target site comprises a base sequence 5'NNGK3', wherein NNG is the at least one triplet.

In another aspect, the invention provides methods of designing a zinc  
15 finger protein. Such methods use a database comprising designations for a plurality of zinc finger proteins, each protein comprising at least first, second and third fingers, and subdesignations for each of the three fingers of each of the zinc finger proteins; a corresponding nucleic acid sequence for each zinc finger protein, each sequence comprising at least first, second and third triplets specifically bound by the at least first,  
20 second and third fingers respectively in each zinc finger protein, the first, second and third triplets being arranged in the nucleic acid sequence (3'-5') in the same respective order as the first, second and third fingers are arranged in the zinc finger protein (N-terminal to C-terminal). A target site is provided for design of a zinc finger protein, the target site comprising continuous first, second and third triplets in a 3'-5' order. For the  
25 first, second and third triplet in the target site, first, second and third sets of zinc finger protein(s) in the database are identified, the first set comprising zinc finger protein(s) comprising a finger specifically binding to the first triplet in the target site, the second set comprising zinc finger protein(s) comprising a finger specifically binding to the second triplet in the target site, the third set comprising zinc finger protein(s) comprising a finger  
30 specifically binding to the third triplet in the target site. Designations and subdesignations of the zinc finger proteins in the first, second, and third sets identified in step (c) are then output. Some method further comprise producing a zinc finger protein that binds to the target site comprising a first finger from a zinc finger protein from the



first set, a second finger from a zinc finger protein from the second set, and a third finger from a zinc finger protein from the third set

Some methods further comprises identifying subsets of the first, second and third sets. The subset of the first set comprising zinc finger protein(s) comprising a  
5 finger that specifically binds to the first triplet in the target site from the first finger position of a zinc finger protein in the database. The subset of the second set comprising zinc finger protein(s) comprises a finger that specifically binds to the second triplet in the target site from the second finger position in a zinc finger protein in the database; the subset of the third set comprises a zinc finger protein(s) comprising a finger that  
10 specifically binds to the third triplet in the target site from a third finger position in a zinc finger protein in the database. Designations and subdesignations of the subset of the first, second and third sets are output. A zinc finger protein comprising a first finger from the first subset, a second finger from the second subset, and a third finger from the third subset is then produced. In some of the above methods of design, the target site is  
15 provided by user input. In some methods, the target site is provided by one of the target site selection methods described above.

The invention further provides computer program products for implementing any of the methods described above. One computer program product implements methods for selecting a target site within a polynucleotide for targeting by a  
20 zinc finger protein. Such a product comprises (a) code for providing a polynucleotide sequence; (b) code for selecting a potential target site within the polynucleotide sequence; the potential target site comprising first, second and third triplets of bases at first, second and third positions in the potential target site; (c) code for calculating a score for the potential target site from a combination of subscores for the first, second,  
25 and third triplets, the subscores being obtained from a correspondence regime between triplets and triplet position, wherein each triplet has first, second and third corresponding positions, and each corresponding triplet and position has a particular subscore; (d) code for repeating steps (b) and (c) at least once on a further potential target site comprising first, second and third triplets at first, second and third positions of the further potential  
30 target site to determine a further score; e) code for providing output of at least one of the potential target site with its score; and (f) a computer readable storage medium for holding the codes.

The invention further provides computer systems for implementing any of the methods described above. One such system for selecting a target site within a polynucleotide for targeting by a zinc finger protein, comprises (a) a memory; (b) a system bus; and (c) a processor. The processor is operatively disposed to: (1) provide or receive a polynucleotide sequence; (2) select a potential target site within the polynucleotide sequence; the potential target site comprising first, second and third triplets of bases at first, second and third positions in the potential target site; (3) calculate a score for the potential target site from a combination of subscores for the first, second, and third triplets, the subscores being obtained from a correspondence regime between triplets and triplet position, wherein each triplet has first, second and third corresponding positions, and each corresponding triplet and position has a particular subscore; (4) repeat steps (2) and (3) at least once on a further potential target site comprising first, second and third triplets at first, second and third positions of the further potential target site to determine a further score; (5) provide output of at least one of the potential target site with its score

A further computer program product for producing a zinc finger protein comprises: (a) code for providing a database comprising designations for a plurality of zinc finger proteins, each protein comprising at least first, second and third fingers; subdesignations for each of the three fingers of each of the zinc finger proteins; a corresponding nucleic acid sequence for each zinc finger protein, each sequence comprising at least first, second and third triplets specifically bound by the at least first, second and third fingers respectively in each zinc finger protein, the first, second and third triplets being arranged in the nucleic acid sequence (3'-5') in the same respective order as the first, second and third fingers are arranged in the zinc finger protein (N-terminus to C-terminus); (b) code for providing a target site for design of a zinc finger protein, the target site comprising at least first, second and third triplets; (c) for the first, second and third triplet in the target site, code for identifying first, second and third sets of zinc finger protein(s) in the database, the first set comprising zinc finger protein(s) comprising a finger specifically binding to the first triplet in the target site, the second set comprising a finger specifically binding to the second triplet in the target site, the third set comprising a finger specifically binding to the third triplet in the target site; (d) code for outputting designations and subdesignations of the zinc finger proteins in the first,

second, and third sets identified in step (c) and, (e) a compute readable storage medium for holding the codes.

The invention further provides a system for producing a zinc finger protein. The system comprises (a) a memory; (b) a system bus; and (c) a processor.

5 The processor is operatively disposed to: (1) provide a database comprising designations for a plurality of zinc finger proteins, each protein comprising at least first, second and third fingers, subdesignations for each of the three fingers of each of the zinc finger proteins; a corresponding nucleic acid sequence for each zinc finger protein, each sequence comprising at least first, second and third triplets specifically bound by the at

10 least first, second and third fingers respectively in each zinc finger protein, the first, second and third triplets being arranged in the nucleic acid sequence (3'-5') in the same respective order as the first, second and third fingers are arranged in the zinc finger protein (N-terminus to C-terminus); (2) provide a target site for design of a zinc finger protein, the target site comprising at least first, second and third triplets, (3) for the first,

15 second and third triplet in the target site, identify first, second and third sets of zinc finger protein(s) in the database, the first set comprising zinc finger protein(s) comprising a finger specifically binding to the first triplet in the target site, the second set comprising a finger specifically binding to the second triplet in the target site, the third set comprising a finger specifically binding to the third triplet in the target site; and (4) output

20 designations and subdesignations of the zinc finger proteins in the first, second, and third sets identified in step (3).

#### BRIEF DESCRIPTION OF THE FIGURES

Fig. 1 shows a chart providing data that the presence and number of sub

25 sites in a target site bound by a zinc finger protein correlates with binding affinity.

Fig. 2 shows a three finger zinc finger protein bound to a target site containing three D-able subsites.

Fig. 3 shows the process of assembling a nucleic acid encoding a designed ZFP.

30 Figs 4 and 5 show computer systems for implementing methods of target site selection and zinc finger protein design

Fig. 6 shows a flow chart of a method for selecting a target site containing a D-able subsite within a target sequence

Fig. 7A shows a flow chart for selecting a target site within a target sequence using a correspondence regime.

Fig. 7B shows a flow chart for designing a ZFP to bind a desired target site using a database.

5 Fig. 8A is an entity representation diagram of a ZFP database.

Fig. 8B is a representation of a ZFP database.

### DEFINITIONS

10 A zinc finger DNA binding protein is a protein or segment within a larger protein that binds DNA in a sequence-specific manner as a result of stabilization of protein structure through coordination on of zinc ion. The term zinc finger DNA binding protein is often abbreviated as zinc finger protein or ZFP.

15 A designed zinc finger protein is a protein not occurring in nature whose design/composition results principally from rational criteria. Rational criteria for design include application of substitution rules and computerized algorithms for processing information in a database storing information of existing ZFP designs and binding data.

A selected zinc finger protein is a protein not found in nature whose production results primarily from an empirical process such as phage display.

20 The term naturally-occurring is used to describe an object that can be found in nature as distinct from being artificially produced by man. For example, a polypeptide or polynucleotide sequence that is present in an organism (including viruses) that can be isolated from a source in nature and which has not been intentionally modified by man in the laboratory is naturally-occurring. Generally, the term naturally-occurring refers to an object as present in a non-pathological (undiseased) individual, such as would  
25 be typical for the species.

A nucleic acid is operably linked when it is placed into a functional relationship with another nucleic acid sequence. For instance, a promoter or enhancer is operably linked to a coding sequence if it increases the transcription of the coding sequence. Operably linked means that the DNA sequences being linked are typically  
30 contiguous and, where necessary to join two protein coding regions, contiguous and in reading frame. However, since enhancers generally function when separated from the promoter by up to several kilobases or more and intronic sequences may be of variable lengths, some polynucleotide elements may be operably linked but not contiguous.

A specific binding affinity between, for example, a ZFP and a specific target site means a binding affinity of at least  $1 \times 10^6 \text{ M}^{-1}$ .

The terms "modulating expression" "inhibiting expression" and "activating expression" of a gene refer to the ability of a zinc finger protein to activate or inhibit transcription of a gene. Activation includes prevention of subsequent transcriptional inhibition (i.e., prevention of repression of gene expression) and inhibition includes prevention of subsequent transcriptional activation (i.e., prevention of gene activation). Modulation can be assayed by determining any parameter that is indirectly or directly affected by the expression of the target gene. Such parameters include, e.g., changes in RNA or protein levels, changes in protein activity, changes in product levels, changes in downstream gene expression, changes in reporter gene transcription (luciferase, CAT, beta-galactosidase, GFP (see, e.g., Mistili & Spector, *Nature Biotechnology* 15:961-964 (1997))); changes in signal transduction, phosphorylation and dephosphorylation, receptor-ligand interactions, second messenger concentrations (e.g., cGMP, cAMP, IP3, and Ca<sup>2+</sup>), cell growth, neovascularization, *in vitro*, *in vivo*, and *ex vivo*. Such functional effects can be measured by any means known to those skilled in the art, e.g., measurement of RNA or protein levels, measurement of RNA stability, identification of downstream or reporter gene expression, e.g., via chemiluminescence, fluorescence, colorimetric reactions, antibody binding, inducible markers, ligand binding assays; changes in intracellular second messengers such as cGMP and inositol triphosphate (IP3); changes in intracellular calcium levels; cytokine release, and the like.

A "regulatory domain" refers to a protein or a protein subsequence that has transcriptional modulation activity. Typically, a regulatory domain is covalently or non-covalently linked to a ZFP to modulate transcription. Alternatively, a ZFP can act alone, without a regulatory domain, or with multiple regulatory domains to modulate transcription.

A D-able subsite within a target site has the motif 5'NNGK3'. A target site containing one or more such motifs is sometimes described as a D-able target site. A zinc finger appropriately designed to bind to a D-able subsite is sometimes referred to as a D-able finger. Likewise a zinc finger protein containing at least one finger designed or selected to bind to a target site including at least one D-able subsite is sometimes referred to as a D-able zinc finger protein.

DETAILED DESCRIPTIONI. General

In one aspect, the invention is directed to methods of selecting appropriate segments within a preselected target gene for design of a zinc finger protein intended for use in modulating or detecting the gene. The size of a potential target gene can vary widely from around 100 to several 100,000 bp. A zinc finger protein can bind to a small subsequence or target site within such gene. For example, zinc finger proteins containing three fingers typically bind to nine or ten bases of a target gene. The invention provides criteria and methods for selecting optimum subsequence(s) from a target gene for targeting by a zinc finger protein.

Some of the methods of target site selection seek to identify one or more target segments having a DNA motif containing one or more so-called D-able subsites. A D-able subsite is defined by a characteristic DNA sequence formula as discussed in detail below. A zinc finger protein is able to bind such a motif in a manner such that at least one component finger of the zinc finger protein contacts an additional base outside the three base subsite usually bound by a finger. If two D-able sites are present in the target segment, then two component fingers of a zinc finger protein can each bind to four bases of the target site. If three D-able subsites are present in the target segment, then three component fingers of zinc finger protein can each bind to four bases in the target site. In general zinc finger proteins binding to target sites containing at least one D-able subsite show higher binding affinity than zinc finger proteins that bind to target segments lacking a D-able subsite. Likewise, zinc finger proteins binding to a target site with two D-able subsites generally show higher binding affinity than zinc finger proteins that bind to a target site with one D-able subsite, and zinc finger proteins with three D-able subsites generally show higher binding affinity than zinc finger proteins that bind to a target site with two D-able subsites. Although an understanding of mechanism is not required for practice of the invention, it is believed that the higher binding affinity results from the additional interactions possible between a zinc finger and four bases in a target segment relative to the interactions possible between a zinc finger and three bases in a target segment. In general, the potential for high affinity binding of target segments with D-able subsites makes them the target sites of choice from within target genes for design of zinc finger proteins because higher binding affinity often results in a greater extent of, and/or greater specificity in, modulation of a target gene.

Other methods of the invention are directed to selection of target segments within target genes by additional or alternative criteria to the D-able subsite. The principal criteria for selection of target segments in such methods are provided in the form of a correspondence regime between different triplets of three bases and the three possible positions of a triplet within a nine-base site (i.e., bases 1-3, 4-6 and 7-9). An exemplary correspondence regime is shown in Table 1. The correspondence regime provides different values for different combinations of triplet and triplet position within a target site. A potential target site within a target gene is evaluated by determining a score for the site by combining subscores for its component triplets obtained from the correspondence regime. The scores of different potential target sites are compared, with a high score indicating desirability of a particular segment as a target site for design of zinc finger binding protein.

In another aspect, the invention provides methods of designing zinc finger proteins that bind to a preselected target site. These methods can, of course, be used following the preselection of target sites according to the procedures and criteria described above. The methods of design use a database containing information about previously characterized zinc finger proteins. This information includes names or other designations of previously characterized zinc finger proteins, the amino acid sequence of their component fingers, and the nucleotide triplets bound by each finger of the proteins. Information in the database is accessed using an algorithm that allows one to select fingers from different previous designs for combination in a novel zinc finger protein having specificity for a chosen target site.

## II. Zinc Finger Proteins

Zinc finger proteins are formed from zinc finger components. For example, zinc finger proteins can have one to thirty-seven fingers, commonly having 2, 3, 4, 5 or 6 fingers. A zinc finger protein recognizes and binds to a target site (sometimes referred to as a target segment) that represents a relatively small subsequence within a target gene. Each component finger of a zinc finger protein can bind to a subsite within the target site. The subsite includes a triplet of three contiguous bases all on the same strand (sometimes referred to as the target strand). The subsite may or may not also include a fourth base on the opposite strand that is the complement of the base immediately 3' of the three contiguous bases on the target strand. In many zinc finger

proteins, a zinc finger binds to its triplet subsite substantially independently of other fingers in the same zinc finger protein. Accordingly, the binding specificity of zinc finger protein containing multiple fingers is usually approximately the aggregate of the specificities of its component fingers. For example, if a zinc finger protein is formed  
 5 from first, second and third fingers that individually bind to triplets XXX, YYY, and ZZZ, the binding specificity of the zinc finger protein is 3'XXX YYY ZZZ5'.

The relative order of fingers in a zinc finger protein from N-terminal to C-terminal determines the relative order of triplets in the 3' to 5' direction in the target. For example, if a zinc finger protein comprises from N-terminal to C-terminal the first,  
 10 second and third fingers mentioned above, then the zinc finger protein binds to the target segment 3'XXXYYYZZZ5'. If the zinc finger protein comprises the fingers in another order, for example, second finger, first finger, third finger, then the zinc finger protein binds to a target segment comprising a different permutation of triplets, in this example, 3'YYYXXXZZZ5' (see Berg & Shi, *Science* 271, 1081-1086 (1996)). The assessment of  
 15 binding properties of a zinc finger protein as the aggregate of its component fingers is, however, only approximate, due to context-dependent interactions of multiple fingers binding in the same protein.

Two or more zinc finger proteins can be linked to have a target specificity that is the aggregate of that of the component zinc finger proteins (see e.g., Kim & Pabo, *PNAS* 95, 2812-2817 (1998)). For example, a first zinc finger protein having first, second  
 20 and third component fingers that respectively bind to XXX, YYY and ZZZ can be linked to a second zinc finger protein having first, second and third component fingers with binding specificities, AAA, BBB and CCC. The binding specificity of the combined first and second proteins is thus 3'XXXYYYZZZ\_\_AAABBBCCC5', where the underline  
 25 indicates a short intervening region (typically 0-5 bases of any type). In this situation, the target site can be viewed as comprising two target segments separated by an intervening segment.

Linkage can be accomplished using any of the following peptide linkers. T G E K P: (Liu et al., 1997, supra.); (G4S)<sub>n</sub> (Kim et al., *PNAS* 93, 1156-1160 (1996).);  
 30 GGRRGGGS; LRQRDGERP; LRQKDGGGSERP; LRQKD(G3S)<sub>2</sub> ERP. Alternatively, flexible linkers can be rationally designed using computer program capable of modeling both DNA-binding sites and the peptides themselves or by phage display methods. In a further variation, noncovalent linkage can be achieved by fusing two zinc finger proteins



with domains promoting heterodimer formation of the two zinc finger proteins. For example, one zinc finger protein can be fused with fos and the other with jun (see Barbas et al., WO 95/119431).

Linkage of two zinc finger proteins is advantageous for conferring a unique binding specificity within a mammalian genome. A typical mammalian diploid genome consists of  $3 \times 10^9$  bp. Assuming that the four nucleotides A, C, G, and T are randomly distributed, a given 9 bp sequence is present  $\sim 23,000$  times. Thus a ZFP recognizing a 9 bp target with absolute specificity would have the potential to bind to  $\sim 23,000$  sites within the genome. An 18 bp sequence is present once in  $3.4 \times 10^{10}$  bp, or about once in a random DNA sequence whose complexity is ten times that of a mammalian genome.

A component finger of zinc finger protein typically contains about 30 amino acids and has the following motif (N-C) :

Cys- (X)<sub>2-4</sub>-Cys-X.X.X.X.X.X.X.X.X.X.X.X.X-**His**-(X)<sub>3-5</sub>-His

15                      -1 1 2 3 4 5 6 7

The two invariant histidine residues and two invariant cysteine residues in a single beta turn are co-ordinated through zinc (see, e.g., Berg & Shi, *Science* 271, 1081-1085 (1996)). The above motif shows a numbering convention that is standard in the field for the region of a zinc finger conferring binding specificity. The amino acid on the left (N-terminal side) of the first invariant His residues is assigned the number +6, and other amino acids further to the left are assigned successively decreasing numbers. The alpha helix begins at residue 1 and extends to the residue following the second conserved histidine. The entire helix is therefore of variable length, between 11 and 13 residues.

The process of designing or selecting a nonnaturally occurring or variant ZFP typically starts with a natural ZFP as a source of framework residues. The process of design or selection serves to define nonconserved positions (i.e., positions -1 to +6) so as to confer a desired binding specificity. One suitable ZFP is the DNA binding domain of the mouse transcription factor Zif268. The DNA binding domain of this protein has the amino acid sequence:

30 YACPVESCDRRFSRSELTTRHIRHTGQKP (F1)

FQCRICMRNFSRSDHLTTHIRHTGEKP (F2)

FACDICGRKFARSDEKRTKIHLRQK (F3)

and binds to a target 5' GCG TGG GCG 3'.

Another suitable natural zinc finger protein as a source of framework residues is Sp-1. The Sp-1 sequence used for construction of zinc finger proteins corresponds to amino acids 531 to 624 in the Sp-1 transcription factor. This sequence is 94 amino acids in length. The amino acid sequence of Sp-1 is as follows

5 PGKKKQHICHIQGGCKVYGKTSHLRAHLRWHTGERP  
FMCTWSYCGKRFTSRDELQRHKRTHHTGEKK  
FACPECPKRFMRSDHLSKHIKTHQNKKG

Sp-1 binds to a target site 5'GGG GCG GGG3'.

An alternate form of Sp-1, an Sp-1 consensus sequence, has the following  
10 amino acid sequence:  
meklrngsgd  
PGKKKQHACPECCKSFSKSSHLRAHQRTHTGERP  
YKCPECCKSFSRDELQRHQRTHTGEKP  
YKCPECCKSFSRSDHLSKHQRTHTQNKKG (lower case letters are a leader sequence  
15 from Shi & Berg, *Chemistry and Biology* 1, 83-89. (1995). The optimal binding sequence for the Sp-1 consensus sequence is 5'GGGGCGGGG3'. Other suitable ZFPs are described below.

There are a number of substitution rules that assist rational design of some zinc finger proteins (see Desjarlais & Berg, *PNAS* 90, 2256-2260 (1993); Choo & Klug,  
20 *PNAS* 91, 11163-11167 (1994); Desjarlais & Berg, *PNAS* 89, 7345-7349 (1992); Jamieson et al., supra; Choo et al., WO 98/53057, WO 98/53058; WO 98/53059; WO 98/53060). Many of these rules are supported by site-directed mutagenesis of the three-finger domain of the ubiquitous transcription factor, Sp-1 (Desjarlais and Berg, 1992; 1993). One of these rules is that a 5' G in a DNA triplet can be bound by a zinc finger  
25 incorporating arginine at position 6 of the recognition helix. Another substitution rule is that a G in the middle of a subsite can be recognized by including a histidine residue at position 3 of a zinc finger. A further substitution rule is that asparagine can be incorporated to recognize A in the middle of triplet, aspartic acid, glutamic acid, serine or threonine can be incorporated to recognize C in the middle of triplet, and amino acids  
30 with small side chains such as alanine can be incorporated to recognize T in the middle of triplet. A further substitution rule is that the 3' base of triplet subsite can be recognized by incorporating the following amino acids at position -1 of the recognition helix: arginine to recognize G, glutamine to recognize A, glutamic acid (or aspartic acid) to

recognize C, and threonine to recognize T. Although these substitution rules are useful in designing zinc finger proteins they do not take into account all possible target sites. Furthermore, the assumption underlying the rules, namely that a particular amino acid in a zinc finger is responsible for binding to a particular base in a subsite is only  
5 approximate. Context-dependent interactions between proximate amino acids in a finger or binding of multiple amino acids to a single base or vice versa can cause variation of the binding specificities predicted by the existing substitution rules.

The technique of phage display provides a largely empirical means of generating zinc finger proteins with a desired target specificity (see e.g., Rebar, US  
10 5,789,538; Choo et al., WO 96/06166; Barbas et al., WO 95/19431 and WO 98/543111; Jamieson et al., supra). The method can be used in conjunction with, or as an alternative to rational design. The method involves the generation of diverse libraries of mutagenized zinc finger proteins, followed by the isolation of proteins with desired DNA-binding properties using affinity selection methods. To use this method, the experimenter  
15 typically proceeds as follows. First, a gene for a zinc finger protein is mutagenized to introduce diversity into regions important for binding specificity and/or affinity. In a typical application, this is accomplished via randomization of a single finger at positions -1, +2, +3, and +6, and sometimes accessory positions such as +1, +5, +8 and +10. Next, the mutagenized gene is cloned into a phage or phagemid vector as a fusion with gene III  
20 of a filamentous phage, which encodes the coat protein pIII. The zinc finger gene is inserted between segments of gene III encoding the membrane export signal peptide and the remainder of pIII, so that the zinc finger protein is expressed as an amino-terminal fusion with pIII or in the mature, processed protein. When using phagemid vectors, the mutagenized zinc finger gene may also be fused to a truncated version of gene III  
25 encoding, minimally, the C-terminal region required for assembly of pIII into the phage particle. The resultant vector library is transformed into *E. coli* and used to produce filamentous phage which express variant zinc finger proteins on their surface as fusions with the coat protein pIII. If a phagemid vector is used, then this step requires superinfection with helper phage. The phage library is then incubated with target DNA  
30 site, and affinity selection methods are used to isolate phage which bind target with high affinity from bulk phage. Typically, the DNA target is immobilized on a solid support, which is then washed under conditions sufficient to remove all but the tightest binding phage. After washing, any phage remaining on the support are recovered via elution

under conditions which disrupt zinc finger – DNA binding. Recovered phage are used to infect fresh *E. coli.*, which is then amplified and used to produce a new batch of phage particles. Selection and amplification are then repeated as many times as is necessary to enrich the phage pool for tight binders such that these may be identified using sequencing and/or screening methods. Although the method is illustrated for pIII fusions, analogous principles can be used to screen ZFP variants as pVIII fusions.

Zinc finger proteins are often expressed with a heterologous domain as fusion proteins. Common domains for addition to the ZFP include, e.g., transcription factor domains (activators, repressors, co-activators, co-repressors), silencers, oncogenes (e.g., myc, jun, fos, myb, max, mad, rel, ets, bcl, myb, mos family members etc.); DNA repair enzymes and their associated factors and modifiers; DNA rearrangement enzymes and their associated factors and modifiers; chromatin associated proteins and their modifiers (e.g. kinases, acetylases and deacetylases); and DNA modifying enzymes (e.g., methyltransferases, topoisomerases, helicases, ligases, kinases, phosphatases, polymerases, endonucleases) and their associated factors and modifiers. A preferred domain for fusing with a ZFP when the ZFP is to be used for repressing expression of a target gene is a the KRAB repression domain from the human KOX-1 protein (Thiesen et al., *New Biologist* 2, 363-374 (1990); Margolin et al., *Proc. Natl. Acad. Sci. USA* 91, 4509-4513 (1994); Pengue et al., *Nucl. Acids Res.* 22:2908-2914 (1994); Witzgall et al., *Proc. Natl. Acad. Sci. USA* 91, 4514-4518 (1994). Preferred domains for achieving activation include the HSV VP16 activation domain (see, e.g., Hagmann et al., *J. Virol.* 71, 5952-5962 (1997)) nuclear hormone receptors (see, e.g., Torchia et al., *Curr. Opin. Cell. Biol.* 10:373-383 (1998)); the p65 subunit of nuclear factor kappa B (Bitko & Barik, *J. Virol.* 72:5610-5618 (1998) and Doyle & Hunt, *Neuroreport* 8:2937-2942 (1997)); Liu et al., *Cancer Gene Ther.* 5:3-28 (1998)), or artificial chimeric functional domains such as VP64 (Seifpal et al., *EMBO J.* 11, 4961-4968 (1992)).

An important factor in the administration of polypeptide compounds, such as the ZFPs, is ensuring that the polypeptide has the ability to traverse the plasma membrane of a cell, or the membrane of an intra-cellular compartment such as the nucleus. Cellular membranes are composed of lipid-protein bilayers that are freely permeable to small, nonionic lipophilic compounds and are inherently impermeable to polar compounds, macromolecules, and therapeutic or diagnostic agents. However,

proteins and other compounds such as liposomes have been described, which have the ability to translocate polypeptides such as ZFPs across a cell membrane.

For example, "membrane translocation polypeptides" have amphiphilic or hydrophobic amino acid subsequences that have the ability to act as membrane-  
5 translocating carriers. In one embodiment, homeodomain proteins have the ability to translocate across cell membranes. The shortest internalizable peptide of a homeodomain protein, Antennapedia, was found to be the third helix of the protein, from amino acid position 43 to 58 (*see, e.g., Prochiantz, Current Opinion in Neurobiology* 6:629-634 (1996)). Another subsequence, the h (hydrophobic) domain of signal peptides, was found  
10 to have similar cell membrane translocation characteristics (*see, e.g., Lin et al., J. Biol. Chem.* 270:1 4255-14258 (1995)).

Examples of peptide sequences which can be linked to a ZFP of the invention, for facilitating uptake of ZFP into cells, include, but are not limited to: an 11 amino acid peptide of the tat protein of HIV; a 20 residue peptide sequence which  
15 corresponds to amino acids 84-103 of the p16 protein (*see Fahraeus et al., Current Biology* 6:84 (1996)); the third helix of the 60-amino acid long homeodomain of Antennapedia (Derossi *et al., J. Biol. Chem.* 269:10444 (1994)); the h region of a signal peptide such as the Kaposi fibroblast growth factor (K-FGF) h region (Lin *et al., supra*); or the VP22 translocation domain from HSV (Elliot & O'Hare, *Cell* 88:223-233 (1997)).  
20 Other suitable chemical moieties that provide enhanced cellular uptake may also be chemically linked to ZFPs.

Toxin molecules also have the ability to transport polypeptides across cell membranes. Often, such molecules are composed of at least two parts (called "binary toxins"): a translocation or binding domain or polypeptide and a separate toxin domain or  
25 polypeptide. Typically, the translocation domain or polypeptide binds to a cellular receptor, and then the toxin is transported into the cell. Several bacterial toxins, including *Clostridium perfringens* iota toxin, diphtheria toxin (DT), *Pseudomonas* exotoxin A (PE), pertussis toxin (PT), *Bacillus anthracis* toxin, and pertussis adenylate cyclase (CYA), have been used in attempts to deliver peptides to the cell cytosol as internal or amino-  
30 terminal fusions (Arora *et al., J. Biol. Chem.*, 268:3334-3341 (1993); Perelle *et al., Infect. Immun.*, 61:5147-5156 (1993); Stenmark *et al., J. Cell Biol.* 113:1025-1032 (1991); Donnelly *et al., PNAS* 90:3530-3534 (1993); Carbonetti *et al., Abstr. Annu. Meet. Am. Soc. Microbiol.* 95:295 (1995); Sebo *et al., Infect. Immun.* 63:3851-3857 (1995); Klimpel

*et al.*, *PNAS U.S.A.* 89:10277-10281 (1992); and Novak *et al.*, *J. Biol. Chem.* 267:17186-17193 1992)).

Such subsequences can be used to translocate ZFPs across a cell membrane. ZFPs can be conveniently fused to or derivatized with such sequences.

- 5 Typically, the translocation sequence is provided as part of a fusion protein. Optionally, a linker can be used to link the ZFP and the translocation sequence. Any suitable linker can be used, e.g., a peptide linker.

### III. Selection of Target Gene

- 10 Zinc finger proteins can be used to modulate the expression of any target polynucleotide sequence. The sequence can be for example, genomic, cDNA or RNA or an expressed sequence tag (EST). Typically, the target polynucleotide includes a gene or a fragment thereof. The term gene is used broadly to include, for example, exonic regions, intronic regions, 5'UTRs, 3' UTRs, 5' flanking sequences, 3' flanking
- 15 sequences, promoters, enhancers, transcription start sites, ribosome binding sites, regulatory sites, poly-adenylation sites. Target genes can be cellular, viral or from other sources including purely theoretical sequences. Target gene sequences can be obtained from databases, such as GenBank, the published literature or can be obtained *de novo*. Target genes include genes from pathological viruses and microorganisms for which
- 20 repression of expression can be used to abort infection. Examples of pathogenic viruses include hepatitis (A, B, or C), herpes virus (e.g., VZV, HSV-1, HSV-6, HSV-II, and CMV, Epstein Barr virus), HIV, ebola, adenovirus, influenza virus, flaviviruses, echovirus, rhinovirus, coxsackie virus, cornovirus, respiratory syncytial virus, mumps virus, rotavirus, measles virus, rubella virus, parvovirus, vaccinia virus, HTLV virus,
- 25 dengue virus, papillomavirus, molluscum virus, poliovirus, rabies virus, JC virus and arboviral encephalitis virus. Some examples of pathogenic bacteria include chlamydia, rickettsial bacteria, mycobacteria, staphylococci, treptocci, pneumonococci, meningococci and conococci, klebsiella, proteus, serratia, pseudomonas, legionella, diphtheria, salmonella, bacilli, cholera, tetanus, botulism, anthrax, plague, leptospirosis,
- 30 and Lyme disease bacteria.

Target genes also include genes from human or other mammals that contribute to disease. Some such genes are oncogenes, tumor suppressors or growth factors that contribute to cancer. Examples of oncogenes include hMSH2 (Fishel *et al.*,

*Cell* 75, 1027-1038 (1993)) and hMLH1 (Papadopoulos et al., *Science* 263, 1625-1628 (1994)). Some examples of growth factors include fibroblast growth factor, platelet-derived growth factor, GM-SCF, VEGF, EPO, Erb-B2, and hGH. Other human genes contribute to disease by rendering a subject susceptible to infection by a microorganism or virus. For example, certain alleles of the gene encoding the CCR5 receptor render a subject susceptible to infection by HIV. Other human genes, such as that encoding amyloid precursor protein or ApoE, contribute to other diseases, such as Alzheimer's disease.

Target genes also include genes of human or other mammals that provide defense mechanisms against diseases due to other sources. For example, tumor repressor genes, provide protection against cancer. Expression of such genes is desirable and zinc finger proteins are used to activate expression.

Target genes also include genes that are normally turned off or expressed at low levels but which through activation can be used to substitute for another defective gene present in some individuals. For example, the fetal hemoglobin genes, which are normally inactive in adult humans, can be activated to substitute for the defective beta-globin gene in individuals with sickle cell anemia.

Target genes also include plant genes for which repression or activation leads to an improvement in plant characteristics, such as improved crop production, disease or herbicide resistance. For example, repression of expression of the FAD2-1 gene results in an advantageous increase in oleic acid and decrease in linoleic and linoleic acids.

#### IV. Design of Zinc Finger Proteins To Bind D-able Subsites

##### 1. Methods

The invention provides methods that select a target gene, and identify a target site within the gene containing one to six (or more) D-able subsites. A zinc finger protein can then be synthesized that binds to the preselected site. These methods of target site selection are premised, in part, on the present inventors' recognition that the presence of one or more D-able subsites in a target segment confers the potential for higher binding affinity in a zinc finger protein selected or designed to bind to that site relative to zinc finger proteins that bind to target segments lacking D-able subsites. Experimental evidence supporting this insight is provided in Examples 2-9.

A D-able subsite is a region of a target site that allows an appropriately designed single zinc finger to bind to up to four bases rather than up to three of the target site. Such a zinc finger binds to a triplet of bases on one strand of a double-stranded target segment (target strand) and a fourth base on the other strand (see Fig. 2). For a single zinc finger to bind a four base target segment imposes constraints both on the sequence of the target strand and on the amino acid sequence of the zinc finger. The target site within the target strand should include the "D-able" subsite motif 5'NNGK3', in which N and K are conventional IUPAC-IUB ambiguity codes. A zinc finger for binding to such a site should include an arginine residue at position -1 and an aspartic acid, (or less preferably a glutamic acid) at position +2. The arginine residue at position -1 interacts with the G residue in the D-able subsite. The aspartic acid (or glutamic acid) residue at position +2 of the zinc finger interacts with the opposite strand base complementary to the K base in the D-able subsite. It is the interaction between aspartic acid (symbol D) and the opposite strand base (fourth base) that confers the name D-able subsite. As is apparent from the D-able subsite formula, there are two subtypes of D-able subsites: 5'NNGG3' and 5'NNGT3'. For the former subsite, the aspartic acid or glutamic acid at position +2 of a zinc finger interacts with a C in the opposite strand to the D-able subsite. In the latter subsite, the aspartic acid or glutamic acid at position +2 of a zinc finger interacts with an A in the opposite strand to the D-able subsite. In general, NNGG is preferred over NNGT.

In the design of a zinc finger protein with three fingers, a target site should be selected in which at least one finger of the protein, and preferably, two or three fingers have the potential to bind a D-able subsite in a target site. Such can be achieved by selecting a target site from within a larger target gene having the formula

25                   5'NNx aNy bNzc3', wherein

wherein each of the sets (x, a), (y, b) and (z, c) is either (N, N) or (G, K);

at least one of (x, a), (y, b) and (z, c) is (G, K). and

N and K are IUPAC-IUB ambiguity codes.



In other words, at least one of the three sets (x, a), (y, b) and (z, c) is the set (G, K) meaning that the first position of the set is G and the second position is G or T. Those of the three sets (if any) which are not (G, K) are (N, N) meaning that the first position of the set can be occupied by any nucleotide and the second position of the set can be occupied by any nucleotide. As an example, the set (x, a) can be (G, K) and the sets (y, b) and (z, c) can both be (N, N).

In the formula 5'NNx aNy bNzc3', the triplets of NNx aNy and bNz represent the triplets of bases on the target strand bound by the three fingers in a zinc finger protein. The complements of the highlighted bases are the sites of potential fourth base binding on the nontarget strand. If only one of x, y and z is a G, and this G is followed by a K, the target site includes a single D-able subsite. For example, if only x is G and a is K, the site reads NNG **K**Ny bNz w with the D-able subsite highlighted. If both x and y but not z are G and a and b are K, then the target site has two overlapping D-able subsites as follows: 5'NNG **KNG** *KNz* c3' with one such site being represented in bold and the other in italics. If all three of x, y and z are G and a, b and c are K, then the target segment includes three D-able subsites, as follows 5'NNG **KNG** *KNG* K3', the D-able subsites being represented by bold, italics and underline.

The methods of the invention thus work by selecting a target gene, and systematically searching within the possible subsequences of the gene for target sites conforming to the formula 5'NNx aNy bNzc3', wherein

wherein each of (x, a), (y, b) and (z, c) is (N, N) or (G, K);

at least one of (x, a), (y, b) and (z, c) is (G, K). and

N and K are IUPAC-IUB ambiguity codes.

In some such methods, every possible subsequence of 10 contiguous bases on either strand of a potential target gene is evaluated to determine whether it conforms to the above formula, and, if so, how many D-able sub-sites are present. Typically, such a comparison is performed by computer, and a list of target sites conforming to the formula are output. Optionally, such target sites can be output in different subsets according to how many D-able subsites are present.

In a variation, the methods of the invention identify first and second target segments, each independently conforming to the above formula. The two target segments in such methods are constrained to be adjacent or proximate (i.e., within about 0-5 bases) of each other in the target gene. The strategy underlying selection of proximate target segments is to allow the design of a zinc finger protein formed by linkage of two component zinc finger proteins specific for the first and second target segments respectively. These principles can be extended to select target sites to be bound by zinc finger proteins with any number of component fingers. For example, a suitable target site for a nine-finger protein would have three component segments, each conforming to the above formula.

The target sites identified by the above methods can be subject to further evaluation by other criteria or can be used directly for design or selection (if needed) and production of a zinc finger protein specific for such a site. A further criterion for evaluating potential target sites is proximity to particular regions within a gene. If a zinc finger protein is to be used to repress a cellular gene on its own (i.e., without linking the zinc finger protein to a repressing moiety), then the optimal location appears to be at the site of transcription initiation, or within about 50 bp upstream or downstream, or alternatively within an enhancer element to interfere with the formation of the transcription complex (Kim & Pabo, *J. Biol. Chem.* (1997) or compete for an essential enhancer binding protein. If, however, a ZFP is fused to a functional domain such as the KRAB repressor domain or the VP16 activator domain, the choice of location of the binding site is considerably more flexible and can be outside known regulatory regions. For example, a KRAB domain can repress transcription of a promoter up to at least 3-kb from where KRAB is bound. Thus, target sites can be selected that do not include or overlap segments of significance with target genes, such as regulatory sequences, or polymorphic sites. Other criteria for further evaluating target segments include the prior availability of zinc finger proteins binding to such segments or related segments, and/or ease of designing new zinc finger proteins to bind a given target segment. Implementation of such criteria in the selection process is discussed in further detail below.

Once a target segment has been selected, a zinc finger protein that binds to the segment can be provided by a variety of approaches. The simplest approach is to provide a precharacterized zinc finger protein from an existing collection that is already

known to bind to the target site. However, in many instances, such a zinc finger protein does not exist. An alternative approach uses information in a database of existing zinc finger proteins and binding specificities to design new zinc finger proteins. This approach is described in more detail below. A further approach is to design a zinc finger protein based on substitution rules as discussed above. A still further alternative is to select a zinc finger protein with specificity for a given target by an empirical process such as phage display. In some such methods, each component finger of a zinc finger protein is designed or selected independently of other component fingers. For example, each finger can be obtained from a different pre-existing ZFP, or each finger can be subject to separate randomization and selection.

Once a zinc finger protein has been selected, designed, or otherwise provided to a given target segment, the zinc finger protein or the DNA encoding are synthesized. Exemplary methods for synthesizing and expressing DNA encoding zinc proteins are described below. The zinc finger protein or a polynucleotide encoding it can then be used for modulation of expression, or analysis of the target gene containing the target site to which the zinc finger protein binds.

## 2. D-able Zinc Finger Proteins

A zinc finger protein is described as D-able if it contains a finger that can bind to the fourth base of at least one D-able subsite, that is a polynucleotide sequence 5'NNGK3'. A preferred framework for designing D-able zinc fingers is the human wild type Sp-1 DNA binding domain. The target for the human transcription factor Sp-1 is 5'GGG GCG GGG3', and fingers 1 and 2 of this protein have an R-1 D+2 arrangement. Designed ZFPs can be identical to Sp-1 except in the recognition helix of each of the three fingers, where the sequences are designed to recognize each of the triplets with which they interact. The mouse ZFP Zif268, which binds the site GCG TGG GCG, is also suitable, having the R-1 D+2 arrangement in all three fingers.

Other zinc finger proteins as a source of framework residues for design of zinc finger proteins capable of binding to D-able subsites can be obtained from or derived from ZFPs from several alternative sources. For example, the TTK transcriptional regulatory protein of the fruit fly *Drosophila melanogaster* has been well characterized with regard to both the sequences of its recognition helices and its DNA site. The protein has only two fingers and binds to a six base target, so finger 2 interacts with the first

DNA triplet and finger 1 recognizes the second triplet of the site. The site is 5'AAG GAT3' with a GG type D-able subsite present at the junction of the first and second triplet, and finger 2 has the R-1 D+2 sequence. Other suitable ZFPs are found in the unicellular eukaryote *Saccharomyces cerevisiae*. The ADR gene product is known to regulate expression of the ADH gene by binding within the ADH promoter. As described above for TTK, the ADR ZFP binding domain has two fingers, and binds to a six base target, TTGGAG. The finger 2 recognition helix has the R-1 D+2 sequence, appropriate for a ZFP binding to a target site with a D-able subsite.

#### IV. Selection of Target Sites by a Correspondence Regime

The invention further provides additional or alternative methods for selecting a target site from within a target gene. These methods are premised, in part, on the insights that different three-base subsites (triplets) bound by individual fingers have different desirabilities for zinc finger protein design, that these different desirabilities can be expressed as numerical values, and that the numerical values for the three individual triplets comprising a target site can be combined to give an overall score for the target site. The relative merits of different target sites can be compared from their relative score.

The methods work by providing a polynucleotide sequence typically a gene or cDNA within which one wishes to select a target site for detection or modulation by a ZFP. In practice, one typically provides two sequences for the two strands of a polynucleotide sequence, but for simplicity, the method is illustrated for a single polynucleotide sequence. From within such a polynucleotide sequence, a potential target site of at least 9 bases comprising contiguous first, second and third triplets of bases is selected. The triplets are contiguous in that the first triplet occupies bases 7-9, the second triplet bases 4-6 and the third triplet bases 1-3 of a site, with base 1 in the 5'-3' orientation being designated base 1. This designation of triplets as first, second, and third is arbitrary and could be reversed. However, by designating the first triplet as occupying bases 7-9, the second triplet bases 4-6 and the third triplet bases 1-3, the first, second, and third fingers of a three finger ZFP in an N-C terminal orientation bind to the first, second and third triplets of a target site. Viewed in another manner, the first, second and third fingers in a zinc finger protein order from N terminal to C terminal are respectively specific for the first, second and third triplets in a target site ordered in the 3'-5' orientation.

A subscore is then determined for each triplet from a correspondence regime between triplets and corresponding positions within a target site. An exemplary correspondence regime is provided in Table 1. The correspondence regime is a matrix providing three values for each triplet at its three possible positions within a nine base target site. The table provides three values for each of the 64 possible triplets. For example, consider a potential target site 5'AAA AAG AAC3'. The AAC triplet occurs in the first position (bases 7-9) of the target site and is assigned a subscore of 1 from Table 1. The AAG triplet occurs in the second position of the target site (bases 4-6) and is assigned a subscore of 8. The AAA triplet occurs in the third position of the target site (bases 1-3) and is assigned a subscore of 8. The subscores of the three triplets in the potential target site are then combined, e.g., by multiplication or addition or some other function. For example, multiplication of the three triplet subscores gives a combined score of  $1 \times 8 \times 8 = 64$ .

The process is then repeated for a second potential target site. Subscores are determined for each of the three component triplets of the second potential target site, and a combined score is calculated for the second potential target site. The process can then be repeated for further potential target sites. Optionally, the process can be repeated for every possible contiguous subsequence of at least 9 bases in either strand of a target gene of interest. When scores of all potential target sites of interest have been determined the scores are compared. In general a high score indicates desirability of a target site for design of a ZFP. One or more of the target sites identified with high scores can be outputted together with the score.

The designation of values in the correspondence regime can reflect any criteria that make one triplet subsite more desirable than another for zinc finger protein design or selection. The values in the exemplary correspondence regime of Table 1 reflect availability of previously characterized ZFPs known to bind a given nucleotide triplet. If for a given triplet in a given position of a target site, there exist one or more previously characterized ZFPs that specifically bind to a target segment including the triplet at the given position, then the combination of the triplet and given position is assigned a score of 10. If for a given triplet at a given position, there are no previously characterized ZFPs that specifically bind a target site including the triplet at the given position, but there are one or more previously characterized ZFPs that specifically bind to the triplet at a different position, then the triplet is assigned a score of 8. If for a given

triplet and a given position, there are no previously characterized ZFPs that bind the triplet either at the given position or another position, the triplet and position are assigned a value 1.

The values 10, 8 and 1 are only illustrative, and other values could be used. Furthermore, a more sophisticated assignment of values can be used which also takes into account different binding affinities, specificities and presence of D-able sites, among other factors. In such a scheme, combinations of triplets and positions for which prior ZFPs exist with strong binding affinities are typically given higher values than combinations of triplet and positions for which there are prior ZFPs with lower binding affinities.

The selection of potential target sites within a larger sequence and calculation of scores is typically performed by a suitably programmed computer, which outputs one or more potential target site(s) with their score(s). Optionally, user input can be provided to such a computer to specify how many potential target sites should be output. For example, the user can elect to have n potential target sites with the highest scores output, where n is at the discretion of the user. The user can also specify a threshold score, which must be equaled or exceeded for a potential target site to be output.

In a variation of the above method, a potential target site can be evaluated based both on values in a correspondence table and on the presence of one or more D-able subsites. Such is achieved by user input of a context parameter to provide a scaled score for one or more combinations of triplet and a particular position, if the context of the triplet indicates presence of a D-able subsite. For example, a triplet 5'NNG3' followed by an A does not provide a D-able subsite. However, 5'NNG3' followed by a K does provide a D-able site. The user can elect to input a context parameter that increases the value of the subscore for the 5'NNG3' triplet when 5'NNG3' is followed by a K. The scaled subscore for this triplet is then combined with subscores or scaled subscores for other triplets to give an overall score for a potential target site.

In a further variation, a computer performing the above analysis is programmed to output certain target segments receiving high scores in pairs determined by their physical proximity to each other. Paired target segments both of which receive high scores that occur within about five bases of each other are appropriate targets for the

design of six-finger zinc proteins formed by linkage of two component zinc finger proteins each having three fingers.

Potential target sites identified by the above methods can be subject to further evaluation or can be used directly for design or selection (if needed) and production of zinc finger proteins. Zinc finger proteins can be designed and synthesized to such target sites using the same methods described for potential target segments containing D-able subsites described above.

#### V. Database design of ZFPs

The invention provides methods for design of ZFPs to a preselected target site. These methods are suitable for use in conjunction with the methods of target site selection described above, or by other methods of target site selection.

In designing a new ZFP, it is generally advantageous to make use of information inherent in precharacterized ZFPs and their target sites thereby minimizing the need for *de novo* design or selection. As with target site selection, several factors are involved in this process. Design is facilitated when, for each triplet subsite in a target site, fingers are not only available in existing ZFPs, but such fingers also contact their respective triplet subsites from the same location in the existing proteins as in the proposed design. For example, consider three existing pairs of ZFP and target site: 5'GCG TGG GAC3', bound by a ZFP with fingers F1-F2-F3 (where F3 interacts with GCG, F2 with TGG, and F1 with GAC), 5'AAG GAG GTG3', bound by a ZFP with fingers F4-F5-F6, and 5'CCG TGA GCA3', bound by a ZFP with fingers F7-F8-F9, and a target site 5'GCG GAG GCA3' for which a ZFP is to be designed. In this situation, the novel protein F7-F5-F3 binds to 5'GCG GAG GCA3' with each finger in the novel protein occurring in the same relative position in the novel protein as it did in the database proteins from which it was obtained. This design is advantageous because the analogous environment of each finger in the novel ZFP with that of its previous ZFP means that the finger is likely to bind with similar specificity and affinity in the novel ZFP as in the parent. Thus, the general rule that the binding characteristics of a zinc finger protein are the aggregate of its component fingers is likely to hold.

Novel zinc finger proteins can also be designed from component fingers that are available in existing proteins, but not at the same positions as in the protein to be designed. For example, using the set of existing ZFP-site pairs described above, the

protein F3-F7-F5 can be designed to bind sequence 5'GAG GCA GCG3'. In the novel protein, the fingers occupy different positions than in their respective parental proteins. Although to an approximation a given finger retains its triplet specificity and affinity irrespective of which position it occupies in a ZFP, in practice, contextual effects are more likely to cause changes in specificity and/or affinity of a finger for its triplet subsite when the finger occupies different positions in different zinc finger proteins. Therefore, although ZFPs formed from component fingers occupying different positions than in previously characterized ZFPs typically still bind to the site, the specificity or affinity is sometimes different (typically lower) than expected.

Finally, for preselected target sites including a triplet for which no preexisting finger is available, completely novel fingers can be designed or selected using rules-based approaches or phage display.

The invention provides methods of systematically using a database containing information about existing ZFPs in the design of new ZFPs for a preselected target site according to the principles described above. The organization of a typical database is shown in Table 9. The database typically includes designations for each of a collection of precharacterized ZFPs. The ZFPs can be natural ZFPs or variant ZFPs. The designation can be, for example, the name or a symbol representing each ZFP. The database also includes subdesignations for each of the fingers in a ZFP. Typically, the subdesignations are in the form of amino acid residues occupying selected positions in a finger or fingers. For example, in Table. 9 the subdesignations are the amino acids occupying positions -1 through +6 according to conventional numbering. The database further includes a target nucleic acid segment bound by each zinc finger protein. The nucleic acid segment usually includes three triplets of three bases. The three triplets of bases can be included joined as one sequence or as separate sequences. If bases in a nine base target site are numbered consecutively from the 5' end, a first triplet occupies bases 7-9, a second triplet occupies bases 4-6 and a third triplet occupies bases 1-3. According to this designation of triplet position within a target segment, the first finger of a zinc finger protein (i.e., closest to N-terminus) binds to the first triplet, the second finger to the second triplet, and the third finger to the third triplet. The database can also include additional information such as the binding affinity or dissociation constant of a ZFP for its target site, although such is not essential.



A target site is provided for design of a zinc finger protein using the database. In some methods, the target site is provided by user input. In other methods, the target site is provided as output from any of the methods of target site selection described above. The target site typically comprises at least 9 bases forming at least three triplets. The three component triplets are designated first, second and third triplets respectively occupying bases 7-9, 4-6 and 1-3 of the target site, with the 5' base being assigned as base 1. For the first triplet in the target site, the computer searches the database for a zinc finger protein(s) containing fingers that bind to the triplet. The computer stores records relating to the zinc finger protein(s) thereby identified, and their finger(s) that bind to the first triplet. Optionally, the computer distinguishes between zinc finger proteins containing a finger that binds to the first triplet of the target site at the first finger position and in other positions. If so, the computer stores the two subsets of zinc finger protein(s) as separate records. The process is then repeated for the second triplet in the target site. The computer identifies zinc finger protein(s) containing a finger that specifically binds to the second triplet. Optionally, the computer distinguishes between zinc finger(s) that bind the second triplet from the second position of an existing zinc finger protein or at a different position. Finally, the computer identifies zinc finger protein(s) containing a finger that specifically binds to the third triplet of the target site. Optionally, the computer distinguishes between zinc finger(s) that bind the third triplet from the third position of an existing zinc finger protein or from another position. After searching for ZFPs that bind to each of the first, second and third triplets in the target segment, the computer outputs designations for the ZFPs that have been identified and subdesignations of the fingers that bind to the first, second and third triplets. Optionally, the computer provides separate output of a subset of ZFPs that bind the first triplet from the first finger position, and a subset of ZFPs that bind the first triplet from other positions; and corresponding subsets of ZFPs that bind the second triplet from the second finger position and from other positions, and of ZFPs that bind the third triplet from the third finger position and from other positions.

The information output by the computer can be used in the design and synthesis of novel zinc finger proteins that bind to a preselected target. For example, if the output includes a ZFP1 with a finger X that binds the first triplet of the target, ZFP2 that includes a finger Y that binds to the second triplet of the target, and ZFP3 that includes a finger Z that binds to the third triplet of the target, a novel ZFP can be

synthesized comprising the fingers XYZ in that order (N-terminal to C-terminal). If the computer outputs multiple different zinc finger proteins that contain multiple different fingers that bind to a given triplet, the user can select between the fingers depending on whether a finger binds to a particular triplet position from the same position in the database protein as in the ZFP to be designed. For example, a ZFP1 containing fingers XYZ, in which X binds to a first triplet in a target site is generally preferred to a ZFP2 containing fingers ABC, in which finger C binds to the first triplet in a target site. Thus one would typically use finger X rather than C to occupy the first finger position in a ZFP designed to bind the target segment. Often the computer program identifies two ZFPs, each containing a finger that binds a particular triplet, and in each ZFP, the finger occupies the same position in the database protein from which it derives as in the intended design ZFP. In such cases, one often chooses between the two fingers based on the binding affinity for their respective targets, with higher binding affinity being preferred. Optionally, the computer also provides output of proposed amino acid substitutions to one or more fingers for the corresponding triplet(s) bound by the finger(s).

Although database analysis is primarily illustrated for precharacterized zinc finger proteins having three fingers, such databases can alternatively or additionally store information concerning zinc finger proteins with fewer or greater numbers of fingers. Likewise, such databases can be used in the design of zinc finger proteins having fewer or greater than three fingers. For example, some databases of the invention store information concerning ZFPs with only two fingers as well as or instead of information concerning ZFPs with three fingers. ZFPs with only two fingers have corresponding target sites with only two triplets. The information relating to two-finger ZFPs can be used in the design of three-finger ZFPs that bind to nine base target sites in essentially the same manner described above. However, there is no exact correspondence between the relative positions of two fingers in a two-finger protein with the relative positions of three fingers in a three-finger zinc finger protein. This issue can be addressed in two ways. First, all fingers in a two-finger protein can be effectively treated as occupying different positions than fingers in a three-finger protein. Accordingly, if a two finger protein contains a finger that binds to a given triplet, the computer outputs this information and indicates that the finger does not occur at the same position in the database two-finger protein as in the three-finger protein to be designed. Alternatively, the first (N-terminal)

finger in a two-finger protein can be considered the equivalent of either the first or second finger in a three-finger protein. The second finger in a two-finger protein can be considered the equivalent of either the second or third finger in a three-finger protein. Accordingly, if the computer identifies a two finger protein with a first (N-terminal) finger binding to a first triplet in a target site for which a zinc finger protein is to be designed, the computer can output that the two finger protein supplies an appropriate finger and at the same position in the database protein as in the three finger protein to be designed.

## 10 VII. Production of ZFPs

ZFP polypeptides and nucleic acids encoding the same can be made using routine techniques in the field of recombinant genetics. Basic texts disclosing the general methods of use in this invention include Sambrook et al., *Molecular Cloning, A Laboratory Manual* (2nd ed. 1989); Kriegler, *Gene Transfer and Expression: A Laboratory Manual* (1990); and *Current Protocols in Molecular Biology* (Ausubel et al., eds., 1994)). In addition, nucleic acids less than about 100 bases can be custom ordered from any of a variety of commercial sources, such as The Midland Certified Reagent Company (mcrc@oligos.com), The Great American Gene Company (http://www.genco.com), ExpressGen Inc. (www.expressgen.com), Operon Technologies Inc. (Alameda, CA). Similarly, peptides can be custom ordered from any of a variety of sources, such as PeptidoGenic (pkim@ccnet.com), HTI Bio-products, inc. (http://www.htibio.com), BMA Biomedicals Ltd (U.K.), Bio.Synthesis, Inc.

Oligonucleotides can be chemically synthesized according to the solid phase phosphoramidite triester method first described by Beaucage & Caruthers, *Tetrahedron Letts.* 22:1859-1862 (1981), using an automated synthesizer, as described in Van Devanter et al., *Nucleic Acids Res.* 12:6159-6168 (1984). Purification of oligonucleotides is by either denaturing polyacrylamide gel electrophoresis or by reverse phase HPLC. The sequence of the cloned genes and synthetic oligonucleotides can be verified after cloning using, e.g., the chain termination method for sequencing double-stranded templates of Wallace et al., *Gene* 16:21-26 (1981).

Two alternative methods are typically used to create the coding sequences required to express newly designed DNA-binding peptides. One protocol is a PCR-based assembly procedure that utilizes six overlapping oligonucleotides (Fig. 3). Three

oligonucleotides (oligos 1, 3, and 5 in Figure 3) correspond to "universal" sequences that encode portions of the DNA-binding domain between the recognition helices. These oligonucleotides typically remain constant for all zinc finger constructs. The other three "specific" oligonucleotides (oligos 2, 4, and 6 in Fig. 3) are designed to encode the  
5 recognition helices. These oligonucleotides contain substitutions primarily at positions - 1, 2, 3 and 6 on the recognition helices making them specific for each of the different DNA-binding domains.

The PCR synthesis is carried out in two steps. First, a double stranded DNA template is created by combining the six oligonucleotides (three universal, three  
10 specific) in a four cycle PCR reaction with a low temperature annealing step, thereby annealing the oligonucleotides to form a DNA "scaffold." The gaps in the scaffold are filled in by high-fidelity thermostable polymerase, the combination of Taq and Pfu polymerases also suffices. In the second phase of construction, the zinc finger template is amplified by external primers designed to incorporate restriction sites at either end for  
15 cloning into a shuttle vector or directly into an expression vector.

An alternative method of cloning the newly designed DNA-binding proteins relies on annealing complementary oligonucleotides encoding the specific regions of the desired ZFP. This particular application requires that the oligonucleotides be phosphorylated prior to the final ligation step. This is usually performed before setting  
20 up the annealing reactions. In brief, the "universal" oligonucleotides encoding the constant regions of the proteins (oligos 1, 2 and 3 of above) are annealed with their complementary oligonucleotides. Additionally, the "specific" oligonucleotides encoding the finger recognition helices are annealed with their respective complementary oligonucleotides. These complementary oligos are designed to fill in the region which  
25 was previously filled in by polymerase in the above-mentioned protocol. The complementary oligos to the common oligos 1 and finger 3 are engineered to leave overhanging sequences specific for the restriction sites used in cloning into the vector of choice in the following step. The second assembly protocol differs from the initial protocol in the following aspects: the "scaffold" encoding the newly designed ZFP is  
30 composed entirely of synthetic DNA thereby eliminating the polymerase fill-in step, additionally the fragment to be cloned into the vector does not require amplification. Lastly, the design of leaving sequence-specific overhangs eliminates the need for

restriction enzyme digests of the inserting fragment. Alternatively, changes to ZFP recognition helices can be created using conventional site-directed mutagenesis methods.

Both assembly methods require that the resulting fragment encoding the newly designed ZFP be ligated into a vector. Ultimately, the ZFP-encoding sequence is cloned into an expression vector. Expression vectors that are commonly utilized include, but are not limited to, a modified pMAL-c2 bacterial expression vector (New England BioLabs or an eukaryotic expression vector, pcDNA (Promega). The final constructs are verified by sequence analysis.

Any suitable method of protein purification known to those of skill in the art can be used to purify ZFPs of the invention (see, Ausubel, supra, Sambrook, supra). In addition, any suitable host can be used for expression, e.g., bacterial cells, insect cells, yeast cells, mammalian cells, and the like.

Expression of a zinc finger protein fused to a maltose binding protein (MBP-ZFP) in bacterial strain JM109 allows for straightforward purification through an amylose column (NEB). High expression levels of the zinc finger chimeric protein can be obtained by induction with IPTG since the MBP-ZFP fusion in the pMal-c2 expression plasmid is under the control of the tac promoter (NEB). Bacteria containing the MBP-ZFP fusion plasmids are inoculated in to 2xYT medium containing 10 $\mu$ M ZnCl<sub>2</sub>, 0.02% glucose, plus 50  $\mu$ g/ml ampicillin and shaken at 37°C. At mid-exponential growth IPTG is added to 0.3 mM and the cultures are allowed to shake. After 3 hours the bacteria are harvested by centrifugation, disrupted by sonication or by passage through a french pressure cell or through the use of lysozyme, and insoluble material is removed by centrifugation. The MBP-ZFP proteins are captured on an amylose-bound resin, washed extensively with buffer containing 20 mM Tris-HCl (pH 7.5), 200 mM NaCl, 5 mM DTT and 50  $\mu$ M ZnCl<sub>2</sub>, then eluted with maltose in essentially the same buffer (purification is based on a standard protocol from NEB). Purified proteins are quantitated and stored for biochemical analysis.

The dissociation constants of the purified proteins, e.g., K<sub>d</sub>, are typically characterized via electrophoretic mobility shift assays (EMSA) (Buratowski & Chodosh, in *Current Protocols in Molecular Biology* pp. 12.2.1-12.2.7 (Ausubel ed., 1996)). Affinity is measured by titrating purified protein against a fixed amount of labeled double-stranded oligonucleotide target. The target typically comprises the natural binding site sequence flanked by the 3 bp found in the natural sequence and additional,

constant flanking sequences. The natural binding site is typically 9 bp for a three-finger protein and 2 x 9 bp + intervening bases for a six finger ZFP. The annealed oligonucleotide targets possess a 1 base 5' overhang which allows for efficient labeling of the target with T4 phage polynucleotide kinase. For the assay the target is added at a concentration of 1 nM or lower (the actual concentration is kept at least 10-fold lower than the than the expected dissociation constant), purified ZFPs are added at various concentrations, and the reaction is allowed to equilibrate for at least 45 min. In addition the reaction mixture also contains 10 mM Tris (pH 7.5), 100 mM KCl, 1 mM MgCl<sub>2</sub>, 0.1 mM ZnCl<sub>2</sub>, 5 mM DTT, 10% glycerol, 0.02% BSA. (NB: in earlier assays poly d(IC) was also added at 10-100 µg/µl.)

The equilibrated reactions are loaded onto a 10% polyacrylamide gel, which has been pre-run for 45 min in Tris/glycine buffer, then bound and unbound labeled target is resolved by electrophoresis at 150V. (alternatively, 10-20% gradient Tris-HCl gels, containing a 4% polyacrylamide stacker, can be used) The dried gels are visualized by autoradiography or phosphorimaging and the apparent K<sub>d</sub> is determined by calculating the protein concentration that gives half-maximal binding.

The assays can also include determining active fractions in the protein preparations. Active fractions are determined by stoichiometric gel shifts where proteins are titrated against a high concentration of target DNA. Titrations are done at 100, 50, and 25% of target (usually at micromolar levels).

#### IX. Applications of Designed ZFPs

ZFPs that bind to a particular target gene, and the nucleic acids encoding them, can be used for a variety of applications. These applications include therapeutic methods in which a ZFP or a nucleic acid encoding it is administered to a subject and used to modulate the expression of a target gene within the subject (see copending application Townsend & Townsend & Crew Attorney Docket 019496-002200, filed January 12, 1998). The modulation can be in the form of repression, for example, when the target gene resides in a pathological infecting microorganisms, or in an endogenous gene of the patient, such as an oncogene or viral receptor, that is contributing to a diseased state. Alternatively, the modulation can be in the form of activation when activation of expression or increased expression of an endogenous cellular gene can ameliorate a diseased state. For such applications, ZFPs, or more typically, nucleic acids encoding

them are formulated with a pharmaceutically acceptable carrier as a pharmaceutical composition.

Pharmaceutically acceptable carriers are determined in part by the particular composition being administered, as well as by the particular method used to administer the composition. (see, e.g., *Remington's Pharmaceutical Sciences*, 17<sup>th</sup> ed. 1985)). The ZFPs, alone or in combination with other suitable components, can be made into aerosol formulations (i.e., they can be "nebulized") to be administered via inhalation. Aerosol formulations can be placed into pressurized acceptable propellants, such as dichlorodifluoromethane, propane, nitrogen, and the like. Formulations suitable for parenteral administration, such as, for example, by intravenous, intramuscular, intradermal, and subcutaneous routes, include aqueous and non-aqueous, isotonic sterile injection solutions, which can contain antioxidants, buffers, bacteriostats, and solutes that render the formulation isotonic with the blood of the intended recipient, and aqueous and non-aqueous sterile suspensions that can include suspending agents, solubilizers, thickening agents, stabilizers, and preservatives. Compositions can be administered, for example, by intravenous infusion, orally, topically, intraperitoneally, intravesically or intrathecally. The formulations of compounds can be presented in unit-dose or multi-dose sealed containers, such as ampules and vials. Injection solutions and suspensions can be prepared from sterile powders, granules, and tablets of the kind previously described.

The dose administered to a patient should be sufficient to effect a beneficial therapeutic response in the patient over time. The dose is determined by the efficacy and  $K_d$  of the particular ZFP employed, the target cell, and the condition of the patient, as well as the body weight or surface area of the patient to be treated. The size of the dose also is determined by the existence, nature, and extent of any adverse side-effects that accompany the administration of a particular compound or vector in a particular patient

In other applications, ZFPs are used in diagnostic methods for sequence specific detection of target nucleic acid in a sample. For example, ZFPs can be used to detect variant alleles associated with a disease or phenotype in patient samples. As an example, ZFPs can be used to detect the presence of particular mRNA species or cDNA in a complex mixtures of mRNAs or cDNAs. As a further example, ZFPs can be used to quantify copy number of a gene in a sample. For example, detection of loss of one copy

of a p53 gene in a clinical sample is an indicator of susceptibility to cancer. In a further example, ZFPs are used to detect the presence of pathological microorganisms in clinical samples. This is achieved by using one or more ZFPs specific to genes within the microorganism to be detected. A suitable format for performing diagnostic assays employs ZFPs linked to a domain that allows immobilization of the ZFP on an ELISA plate. The immobilized ZFP is contacted with a sample suspected of containing a target nucleic acid under conditions in which binding can occur. Typically, nucleic acids in the sample are labeled (e.g., in the course of PCR amplification). Alternatively, unlabelled probes can be detected using a second labelled probe. After washing, bound-labelled nucleic acids are detected.

ZFPs also can be used for assays to determine the phenotype and function of gene expression. Current methodologies for determination of gene function rely primarily upon either overexpression or removing (knocking out completely) the gene of interest from its natural biological setting and observing the effects. The phenotypic effects observed indicate the role of the gene in the biological system.

One advantage of ZFP-mediated regulation of a gene relative to conventional knockout analysis is that expression of the ZFP can be placed under small molecule control. By controlling expression levels of the ZFPs, one can in turn control the expression levels of a gene regulated by the ZFP to determine what degree of repression or stimulation of expression is required to achieve a given phenotypic or biochemical effect. This approach has particular value for drug development. By putting the ZFP under small molecule control, problems of embryonic lethality and developmental compensation can be avoided by switching on the ZFP repressor at a later stage in mouse development and observing the effects in the adult animal. Transgenic mice having target genes regulated by a ZFP can be produced by integration of the nucleic acid encoding the ZFP at any site *in trans* to the target gene. Accordingly, homologous recombination is not required for integration of the nucleic acid. Further, because the ZFP is trans-dominant, only one chromosomal copy is needed and therefore functional knock-out animals can be produced without backcrossing.

30

#### X. Computer Systems and Programs



Fig. 4 depicts a representative computer system suitable for implementing the present invention. Fig. 4 shows basic subsystems of a computer system 10 suitable for use with the present invention. In Fig. 4, computer system 10 includes a bus 12 which interconnects major subsystems such as a central processor 14, a system memory 16, an input/output controller 18, an external device such as a printer 20 via a parallel port 22, a display screen 24 via a display adapter 26, a serial port 28, a keyboard 30, a fixed disk drive 32 and a floppy disk drive 33 operative to receive a floppy disk 33A. Many other devices can be connected such as a scanner 60 (not shown) via I/O controller 18, a mouse 36 connected to serial port 28 or a network interface 40. Many other devices or subsystems (not shown) may be connected in a similar manner. Also, it is not necessary for all of the devices shown in Fig. 4 to be present to practice the present invention, as discussed below. The devices and subsystems may be interconnected in different ways from that shown in Fig. 4. The operation of a computer system such as that shown in Fig. 4 is readily known in the art and is not discussed in detail in the present application.

Source code to implement the present invention may be operably disposed in system memory 16 or stored on storage media such as a fixed disk 32 or a floppy disk 33A.

Fig. 5 is an illustration of representative computer system 10 of Fig. 4 suitable for embodying the methods of the present invention. Fig. 5 depicts but one example of many possible computer types or configurations capable of being used with the present invention. Fig. 5 shows computer system 10 including display screen 24, cabinet 20, keyboard 30, a scanner 60, and mouse 36. Mouse 36 and keyboard 30 illustrate "user input devices." Other examples of user input devices are a touch screen, light pen, track ball, data glove, etc.

In a preferred embodiment, System 10 includes a Pentium® class based computer, running Windows® Version 3.1, Windows95® or Windows98® operating system by Microsoft Corporation. However, the method is easily adapted to other operating systems without departing from the scope of the present invention.

Mouse 36 may have one or more buttons such as buttons 37. Cabinet 20 houses familiar computer components such as disk drive 33, a processor, storage means, etc. As used in this specification "storage means" includes any storage device used in connection with a computer system such as disk drives, magnetic tape, solid state memory, bubble memory, etc. Cabinet 20 may include additional hardware such as

input/output (I/O) interface 18 for connecting computer system 10 to external devices such as a scanner 60, external storage, other computers or additional peripherals. Fig. 5 is representative of but one type of system for embodying the present invention. Many other system types and configurations are suitable for use in conjunction with the present invention.

Fig. 6 depicts a flowchart 301 of simplified steps in a representative embodiment for selecting a target site containing a D-able subsite within a target sequence for targeting by a zinc finger protein. In a step 302, a target sequence to be targeted by a zinc finger protein is provided. Then, in a step 303, a potential target site within the target sequence is selected for evaluation. In a decisional step 304, the potential target site is evaluated to determine whether it contains a D-able subsite. Such a target site conforms to the formula

5'NNx aNy bNzc3', wherein

wherein each of (x, a), (y, b) and (z, c) is (N, N) or (G, K);

at least one of (x, a), (y, b) and (z, c) is (G, K) and

N and K are IUPAC-IUB ambiguity codes.

If the potential target site does contain a D-able subsite, the potential target site is stored as a record in 205. The method continues with a further decisional step 306. If evaluation of further potential target sites is required by the user, a further iteration of the method is performed starting from 303. If sufficient potential target sites have already been evaluated, records of target sites stored in step 305 are then output in step 307.

Fig. 7A depicts a flowchart of simplified steps in another representative embodiment for selecting a target site within a polynucleotide for targeting by a zinc finger protein. In a step 402, a polynucleotide target sequence is provided for analysis. Then, in a step 404, a potential target site within the polynucleotide sequence is selected. The potential target site comprises first, second and third triplets of bases at first, second and third positions in the potential target site. Then, in a step 406, a plurality of subscores are determined by applying a correspondence regime between triplets and triplet position, wherein each triplet has first, second and third corresponding positions, and each

corresponding triplet and position is assigned a particular subscore. Next there is an optional decisional step 408 in which the user can elect to scale one or more of the subscores with a scaling factor in step 410. Thereafter in a step 412, a score is determined from the subscores (scaled as appropriate) for the first, second, and third triplets. Then, in a decisional step 414, a check is performed to determine if any further potential target sites are to be examined. If so, then processing continues with step 404. Otherwise, in a step 416, at least one of the potential target sites and its score are provided as output.

Fig. 7B depicts a flowchart of simplified steps in a representative embodiment for producing a zinc finger protein. In a step 450 a database comprising designations for a plurality of zinc finger proteins is provided. Each protein in the database comprises at least first, second and third fingers. The database further comprises subdesignations for each of the three fingers of each of the zinc finger proteins and a corresponding nucleic acid sequence for each zinc finger protein. Each sequence comprises at least first, second and third triplets specifically bound by the at least first, second and third fingers respectively in each zinc finger protein. The first, second and third triplets have an arrangement in the nucleic acid sequence in the same respective order (3'-5') as the first, second and third fingers are arranged in the zinc finger protein (N-terminal to C-terminal).

In a step 452, a target site for design of a zinc finger proteins comprising at least first, second and third triplets is provided. Then, in a step 454, a first set of zinc finger proteins with a finger that binds to the first triplet in the target sequence is identified. There follows an optional step 456 of identifying first and second subsets of the set determined in 454. The first subset comprises zinc finger protein(s) with a finger that binds the first triplet from the first finger position in the zinc finger protein. The second subset comprises zinc finger protein(s) with a finger that binds the first triplet from other than the first finger position in the zinc finger protein. The method continues at step 458. In this step, a further set of zinc finger proteins is identified, this set comprising a finger that binds to the second triplet in the target site. This step is followed by an optional step 460 of identifying first and second subsets of the set identified in step 458. The first subset comprises zinc finger protein(s) that bind to the second triplet from the second position within a zinc finger protein. The second subset comprises zinc finger protein(s) that bind the second triplet from other than the second position of a zinc finger

protein. The method continues at step 462. In 462, a set of zinc finger proteins is identified comprising a finger that binds to the third triplet of the target site. In an optional step 464, first and second subsets of the set identified in step 462 are identified. The first subset comprises zinc finger protein(s) containing a finger that binds to the third triplet from the third finger position of the zinc finger protein. The second subset comprises zinc finger protein(s) containing a finger that binds to the third triplet from other than the third finger position of the zinc finger protein. The method continues at step 466 in which the sets of zinc finger protein identified in steps 454, 458 and 462 are separately output. There is a further optional step 468 in which the first and second subsets of zinc finger proteins identified in steps 460, 464 and 468 are output.

Fig. 8A is a key to the Entity Representation Diagram (ERD) that will be used to describe the contents of ZFP database. A representative table 502 includes one or more key attributes 504 and one or more non-key attributes 506. Representative table 502 includes one or more records where each record includes fields corresponding to the listed attributes. The contents of the key fields taken together identify an individual record. In the ERD, each table is represented by a rectangle divided by a horizontal line. The fields or attributes above the line are key while the fields or attributes below the line are non-key fields. An identifying relationship 508 signifies that the key attribute of a parent table 510 is also a key attribute of a child table 512. A non-identifying relationship 514 signifies that the key attribute of a parent table 516 is also a non-key attribute of a child table 518. Where (FK) appears in parenthesis, it indicates that an attribute of one table is a key attribute of another table. For both the non-identifying and the identifying relationships, one record in the parent table corresponds to one or more records in the child table.

Fig. 8B depicts a representative ZFP database 550 according to a particular embodiment of the present invention. Database 550 can typically include designations for each of a collection of precharacterized ZFPs. The ZFPs can be natural ZFPs or variant ZFPs. The designation can be, for example, the name or a symbol representing each ZFP. For example, ZFP 552 of database 550 in Fig. 8B is designated "ZFP001." The database 550 also includes subdesignations for each of the fingers in a ZFP, such as subdesignation 554, Finger 1 of ZFP001 552. Typically, the subdesignations are in the form of amino acid residues occupying selected positions in a finger. Further, the ZFPs have subdesignations that are the amino acids occupying positions -1 through +6

according to conventional numbering. The database can further include a target nucleic acid segment bound by each zinc finger protein. The nucleic acid segment usually includes three triplets of three bases. The three triplets of bases can be included joined as one sequence or as separate sequences. If bases in a nine base target site are numbered consecutively from the 5' end, a first triplet occupies bases 7-9, a second triplet occupies bases 4-6 and a third triplet occupies bases 1-3. According to this designation of triplet position within a target segment, the first finger of a zinc finger protein (i.e., closest to N-terminus) binds to the first triplet, the second finger to the second triplet, and the third finger to the third triplet. The database can also include additional information such as the binding affinity or dissociation constant of a ZFP for its target site, although such is not essential. Further database 550 can include other arrangements and relationships among the ZFPs, fingers and nucleic acids than are depicted in Fig. 8B without departing from the scope of the present invention.

## 15 Examples

### Example 1: SEARCH PROTOCOLS FOR DNA MOTIFS

This Example illustrates how a target segment is selected from a longer gene. The search procedure is implemented using a computer program that allows one to specify one or more DNA sequence motifs in a search protocol. Normal procedure is to input the DNA sequence of a gene or cDNA and then search the sequence multiple times for different motifs, from the most to the least desirable. Thus, of the exemplary protocols listed below one would typically perform protocol 1 first, and if that fails to yield an adequate number of potential target segments, one then tries protocol 2, and so forth.

25 Protocol 1 searches a target gene for a target site formed from two separate segments, each of 9 or 10 bases. The two segments can be separated by zero to three intervening bases. Each segment includes a D-able subsite of the form NNGG (shown in bold). Each three base subsite within a segment begins with a G. The target sites identified by this analysis can be used directly for ZFP design or can be subject to further analysis, for example, to identify which target segments possess additional D-able subsites. In a target site formed from two segments, each of ten bases, a total of six D-able subsites can be present. All target sites below are shown from 5' to 3' and the nomenclature "0,3" indicates that 0-3 nucleotide of any type may be present.

GNGGNNGNN(N){0,3}GNGGNNGNNN  
 GNGGNNGNN(N){0,3}GNNGNGGNNN  
 GNGGNNGNN(N){0,3}GNGGNNGNGG  
 5 GNNGNGGNN(N){0,3}GNGGNNGNNN  
 GNNGNGGNN(N){0,3}GNNGNGGNNN  
 GNNGNGGNN(N){0,3}GNGGNNGNGG  
 GNNGNNGNGG(N){0,3}GNGGNNGNNN  
 GNNGNNGNGG(N){0,3}GNNGNGGNNN  
 10 GNNGNNGNGG(N){0,3}GNGGNNGNGG  
 GNNGNNGNGGNGGNNGNNN  
 GNNGNNGNGGNNGNGGNNN  
 GNNGNNGNGGNNGNNGNGG

15

Protocol 2 is a second procedure for evaluating target sites within a target gene. This procedure again searches for a target site formed from two segments, each of 9 or 10 bases. Each segment contains at least one D-able subsite of the form KNNG. Protocol 2 differs from protocol 1 in that protocol 2 does not require that three base subsites begin with a G. Rather in protocol 2, three base subsites beginning with either a G or T (K in IUBPAC-IUB ambiguity code). Target sites are shown from 5' to 3', and the symbols "(0,3) and (0,2) indicate intervening segments of 0-3 and 0-2 bases respectively.

25

KNGGNNKNN(N){0,3}KNGGNNKNNN  
 KNGGNNKNN(N){0,3}KNNKNGGNNN  
 KNGGNNKNN(N){0,3}KNNKNNKNGG  
 KNNKNGGNN(N){0,3}KNGGNNKNNN

45

KNNKNGGNN(N){0,3}KNNKNGGNNN  
 KNNKNGGNN(N){0,3}KNNKNNKNGG  
 KNNKNNKNGG(N){0,2}KNGGNNKNNN  
 KNNKNNKNGG(N){0,2}KNNKNGGNNN  
 5 KNNKNNKNGG(N){0,2}KNNKNNKNGG  
 KNNKNNKNGGNGGNNKNNN  
 KNNKNNKNGGNNKNGGNNN  
 KNNKNNKNGGNNKNNKNGG

10 Protocol 3 is the same as protocol two except that protocol three selects  
 target sites with either a KNGG or a KNGT D-able subsite. Target sites are shown from  
 5'-3'.

KNGKNNKNN(N){0,3}KNGKNNKNNN  
 15 KNGKNNKNN(N){0,3}KNNKNGKNNN  
 KNGKNNKNN(N){0,3}KNNKNNKNGK  
 KNNKNGKNN(N){0,3}KNGKNNKNNN  
 KNNKNGKNN(N){0,3}KNNKNGKNNN  
 KNNKNGKNN(N){0,3}KNNKNNKNGK  
 20 KNNKNNKNGK(N){0,2}KNGKNNKNNN  
 KNNKNNKNGK(N){0,2}KNNKNGKNNN  
 KNNKNNKNGK(N){0,2}KNNKNNKNGK  
 KNNKNNKNGKNGKNNKNNN  
 KNNKNNKNGKNNKNGKNNN  
 25 KNNKNNKNGKNNKNNKNGK

Protocol 4 is more general than any of the protocols described above, and does not require that target sites contain a D-able subsite. Protocol 4 similar requires two segments, each of 9 bases within 0-3 bases of each other of the form GNN GNN GNN.

Protocol 5 is the same as protocol 4 except that it searches for target sites  
 5 formed from two target segments of formula 5'KNN KNN KNN3' within 0-3 bases of each other.

### Example 2

This example illustrates that zinc finger proteins that bind to target  
 10 segments including at least one D-able subsite generally bind with higher affinity than zinc finger proteins binding to target segments lacking D-able subsites provided the ZFP has a D residues at position +2. Fifty-three ZFPs, each having three fingers, were selected from a collection without regard to binding affinity or binding to a D-able subsite. The dissociation constants of the selected ZFPs were determined by binding of  
 15 the ZFPs to a target segment comprising three contiguous nucleotide triplets respectively bound by the three fingers of the ZFP plus at least one flanking base from the target sequence on either side. All ZFPs had the human Sp1 framework. The binding affinities of these 53 ZFPs were arbitrarily divided into 4 groups, listed as Kd values in Table 2.

20

Table 2

25	Dissociation Constants (Kd)			
	>1,000nM	100-1,000nM	10-99nM	< or =10nM
	31	8	11	3

According to this classification only about 25% (14/53) of these proteins had high  
 30 affinities (Kd less than or equal to 100 nM) for their respective targets. Of these 14 proteins, all had had least one D-able subsite within the target.

### Example 3

35 We searched the sequence of the soybean (Glycine max) FAD2-1 cDNA for paired proximate 9 base target segments using protocols 2 and 3. Five targets segments were chosen, and either one or two ZFPs were designed to bind to each of the targets. The



Table 1: The scores table

5	subsite	subsite score:			subsite	subsite score:		
	<u>sequence:</u>				<u>sequence:</u>			
		location in 9 base site:				location in 9 base site:		
		base pairs #				base pairs #		
10		7-9	4-6	1-3		7-9	4-6	1-3
	AAA	10	8	8	CAA	8	8	10
	AAG	8	8	10	CAG	1	1	1
15	AAC	1	1	1	CAC	1	1	1
	AAT	8	10	10	CAT	1	1	1
	AGA	10	8	8	CGA	1	1	1
	AGG	1	1	1	CGG	1	1	1
	AGC	1	1	1	CGC	1	1	1
20	AGT	1	1	1	CGT	1	1	1
	ACA	8	10	8	CCA	1	1	1
	ACG	1	1	1	CCG	1	1	1
	ACC	1	1	1	CCC	1	1	1
	ACT	1	1	1	CCT	1	1	1
25	ATA	8	10	8	CTA	1	1	1
	ATG	1	1	1	CTG	1	1	1
	ATC	1	1	1	CTC	1	1	1
	ATT	1	1	1	CTT	1	1	1
	GAA	10	10	10	TAA	8	8	10
30	GAG	10	10	10	TAG	10	10	8
	GAC	10	10	8	TAC	10	8	10
	GAT	10	10	10	TAT	1	1	1
	GGA	10	10	10	TGA	10	10	8

58

	GGG	10	10	10		TGG	10	10	10
	GGC	10	10	10		TGC	8	10	10
	GGT	10	10	10		TGT	10	10	8
	GCA	10	10	10		TCA	10	8	8
5	GCG	10	10	10		TCG	8	10	8
	GCC	10	10	8		TCC	10	8	10
	GCT	10	10	10		TCT	1	1	1
	GTA	10	10	10		TTA	10	10	8
	GTG	10	10	10		TTG	8	10	8
10	GTC	10	10	10		TTC	1	1	1
	GTT	10	10	10		TTT	8	10	8

15 Table 9: Exemplary ZFP data table

#	<u>target site</u>	<u>ZFP sequence</u>			<u>reference information</u>
		F1	F2	F3	
20	1 TGC GGGGCA	RSADLTR	RSDHLTR	ERDHLRT	SBS design GR-223, Kd: 8 nM
	2 GCG TGGGCG	RSDELTR	RSDHLTT	RSDERKR	Zif 268, Kd: 0.04 nM
	3 GGG GCGGGG	KTSHLRA	RSDELQR	RSDHLSK	SP1, Kd: 25 nM
	4 GAG TGTGTG	RKDSLVR	TSDHLAS	RSDNLTR	SBS design GL-8.3.1, Kd: 32 nM

25

Other examples of zinc finger proteins, the sequences of their fingers and target sites bound appropriate for inclusion in such a database are discussed in the references cited in the Background Section.

Although the foregoing invention has been described in detail for purposes of clarity of understanding, it will be obvious that certain modifications may be practiced within the scope of the appended claims. All publications and patent documents cited herein are hereby incorporated by reference in their entirety for all purposes to the same extent as if each were so individually denoted.

35

## WHAT IS CLAIMED IS:

Selection of D-able subsites

- 1                   1.     A method of selecting a target site within a target sequence for  
2     targeting by a zinc finger protein comprising:  
3                    providing a target nucleic acid to be targeted by a zinc finger protein;  
4                    outputting a target site within the target nucleic acid comprising 5'NNx  
5     aNy bNzc3', wherein  
  
6                    each of (x, a), (y, b) and (z, c) is (N, N) or (G, K);  
  
7                    at least one of (x, a), (y, b) and (z, c) is (G, K). and  
  
8                    N and K are IUPAC-IUB ambiguity codes.
- 1                   2.     The method of claim 1, further comprising selecting a plurality of  
2     potential target sites within the target nucleic acid and outputting a subset of the plurality  
3     of potential target segments comprising 5'NNx aNy bNzc3', wherein  
  
4                    each of (x, a), (y, b) and (z, c) is (N, N) or (G, K);  
  
5                    at least one of (x, a), (y, b) and (z, c) is (G, K). and  
  
6                    N and K are IUPAC-IUB ambiguity codes.
- 1                   3.     The method of claim 2, wherein the target nucleic acid comprises a  
2     target gene.
- 1                   4.     The method of claim 1, wherein at least two of (x, a), (y, b) and (z,  
2     c) is (G, K).
- 1                   5.     The method of claim 1, wherein all three of (x, a), (y, b) and (z, c)  
2     are (G, K).
- 1                   6.     The method of claim 1, wherein the zinc finger protein comprises  
2     three fingers.

1                   7.     The method of claim 1, wherein the target site comprises first and  
2 second target segments, each comprising 5'NNx aNy bNzc3', and the method further  
3 comprises selecting the second target segment.

1                   8.     The method of claim 7, wherein in the second segment at least two  
2 of (x, a), (y, b) and (z, c) are (G, K).

1                   9.     The method of claim 8, wherein in the second segment all three of  
2 (x, a), (y, b) and (z, c) are (G, K).

1                   10.    The method of claim 10, wherein the first and second target  
2 segment are separated by fewer than 5 bases in the target site.

1                   11.    The method of claim 10, wherein the first target segment comprises  
2 5'NNN NNN NNG3', the second target segment comprises 5'KNx aNY bNzc3' and  
3 there are zero bases separating the first and second target segments in the target site.

1                   12.    The method of claim 7, further comprising synthesizing step  
2 comprises synthesizing a first zinc finger protein comprising three zinc fingers that  
3 respectively bind to the NNx aNy and bNz triplets in the target segment and a second  
4 three fingers that respectively bind to the NNx aNy and bNz triplets in the second target  
5 segment.

1                   13.    The method of claim 1, further comprising synthesizing a zinc  
2 finger protein comprising first, second and third fingers that bind to the bNz aNy and  
3 NNx triplets respectively.

1                   14.    The method of claim 13, wherein each of the first, second and third  
2 fingers is selected or designed independently.

1                   15.    The method of claim 13, wherein a finger is designed from a database  
2 containing designation of zinc finger proteins, subdesignations of finger components, and  
3 nucleic acid sequences bound by the zinc finger proteins.

1                   16.    The method of claim 13, wherein a finger is selected by screening  
2                   variants of a zinc finger binding protein for specific binding to the target site to  
3                   identify a variant that binds to the target site.

4                   17.    The method of claim 13, further comprising contacting a sample  
5                   containing the target nucleic acid with the zinc finger protein, whereby the zinc finger  
6                   protein binds to the target site revealing the presence of the target nucleic acid or a  
7                   particular allelic form thereof.

1                   18.    The method of claim 13, further comprising contacting a sample  
2                   containing the target nucleic acid with the zinc finger protein, whereby the zinc finger  
3                   protein binds to the target site thereby modulating expression of the target nucleic acid

1                   19.    The method of claim 1, wherein the target site occurs in a coding  
2                   region of a gene

1                   20.    The method of claim 1, wherein the target site occurs within or  
2                   proximal to a promoter, enhancer, or transcription start sit

1                   21.    The method of claim 1, wherein the target site occurs outside a  
2                   promoter, regulatory sequence or transcriptional start site within the target nucleic acid.

#### Selection of Target Sites Using a Correspondence Regim

1                   22.    A method for selecting a target site within a polynucleotide for  
2                   targeting by a zinc finger protein, comprising:  
3                   providing a polynucleotide sequence;  
4                   selecting a potential target site of within the polynucleotide  
5                   sequence; the potential target site comprising contiguous first, second and third triplets of  
6                   bases at first, second and third positions in the potential target site;  
7                   determining a plurality of subscores by applying a correspondence regime  
8                   between triplets and triplet position in a sequence of three contiguous triplets, wherein  
9                   each triplet has first, second and third corresponding positions, and each combination of  
10                  triplet and triplet position has a particular subscore

11                   calculating a score for the potential target site by combining subscores for  
12   the first, second, and third triplets;  
13                   repeating the selecting, determining and calculating steps at least once on a  
14   further potential target site comprising first, second and third triplets at first, second and  
15   third positions of the further potential target site to determine a further score;  
16                   providing output of at least one potential target site with its score.

1                   23.    The method of claim 22, wherein output is provided of the  
2   potential target site with the highest score.

1                   24.    The method of claim 22, wherein output is provided of the n  
2   potential target sites with the highest scores, and the method further comprises providing  
3   user input of a value for n.

1                   25.    The method of claim 22, wherein the subscores are combined by  
2   forming the product of the subscores.

1                   26.    The method of claim 25, wherein the correspondence regime  
2   comprises 64 triplets, each having first, second, and third corresponding positions, and  
3   192 subscores.

1                   27.    The method of claim 22, wherein the subscores in the  
2   correspondence regime are determined by assigning a first value as the subscore of a  
3   subset of triplets and corresponding positions, for each of which there is an existing zinc  
4   finger protein that comprising a finger that specifically binds to the triplet from the same  
5   position in the existing zinc finger protein as the corresponding position of the triplet in  
6   the correspondence regime; assigning a second value as the subscore of a subset of  
7   triplets and corresponding positions, for each of which there is an existing zinc finger  
8   protein that comprises a finger that specifically binds to the triplet from a different  
9   position in the existing zinc finger protein than the corresponding position of the triplet in  
10   the correspondence regime; and assigning a third value as the subscore of a subset of  
11   triplets and corresponding positions for which there is no known zinc protein comprising  
12   a finger that specifically binds to the triplet.

- 1                   28.    The method of claim 22, wherein the correspondence regime is  
2 shown in Table 1.
- 1                   29.    The method of claim 22, further comprising combining a context  
2 parameter with the subscore of at least one of the first, second and third triplets to give a  
3 scaled subscore of the at least one triplet.
- 1                   30.    The method of claim 29, wherein the context parameter is  
2 combined with the subscore when the target site comprises a base sequence 5'NNGK3',  
3 wherein NNG is the at least one triplet.
- 1                   31.    The method of claim 22, further comprising combining a context  
2 parameter that is combined with the score of a potential target site to give a scaled score
- 1                   32.    The method of claim 31, wherein the context parameter is  
2 combined with the score when a potential target site comprises 5'NNx aNy bNzc3',  
3 wherein  
4                   wherein each of (x, a), (y, b) and (z, c) is (N, N) or (G, K);  
5                   at least one of (x, a), (y, b) and (z, c) is (G, K). and  
6                   N and K are IUPAC-IUB ambiguity codes.
- 1                   33.    The method of claim 32, wherein a first context parameter is  
2 combined with the score if one of (x, a), (y, b) and (z, c) is (G, K), and a second context  
3 parameter is combined with the score if two of (x, a), (y, b) and (z, c) are (G, K), and a  
4 third context parameter is input if three of (x, a), (y, b) and (z, c) are (G, K)
- 1                   34.    The method of claim 22, wherein output is provided of at least a  
2 nonoverlapping pair of potential target sites and their scores, the members of the pair  
3 being separated by five or fewer bases in the polynucleotide.

#### Design of ZFPs using a Database

- 1                   35.    A method of producing a zinc finger protein comprising:

- 2 (a) providing a database comprising designations for a plurality of zinc  
3 finger proteins, each protein comprising at least first, second and third fingers, and  
4 subdesignations for each of the three fingers of each of the zinc finger proteins;  
5 a corresponding nucleic acid sequence for each zinc finger protein, each  
6 sequence comprising at least first, second and third triplets specifically bound by the at  
7 least first, second and third fingers respectively in each zinc finger protein, the first,  
8 second and third triplets being arranged in the nucleic acid sequence (3'-5') in the same  
9 respective order as the first, second and third fingers are arranged in the zinc finger  
10 protein (N-terminal to C-terminal);  
11 (b) providing a target site for design of a zinc finger protein, the target site  
12 comprising continuous first, second and third triplets in a 3'-5' order,  
13 (c) for the first, second and third triplet in the target site, identifying first,  
14 second and third sets of zinc finger protein(s) in the database, the first set comprising zinc  
15 finger protein(s) comprising a finger specifically binding to the first triplet in the target  
16 site, the second set comprising zinc finger protein(s) comprising a finger specifically  
17 binding to the second triplet in the target site, the third set comprising zinc finger  
18 protein(s) comprising a finger specifically binding to the third triplet in the target site;  
19 (d) outputting designations and subdesignations of the zinc finger proteins  
20 in the first, second, and third sets identified in step (c).

1 36. The method of claim 35, further comprising:

- 2 (e) producing a zinc finger protein that binds to the target site comprising  
3 a first finger from a zinc finger protein from the first set, a second finger from a zinc  
4 finger protein from the second set, and a third finger from a zinc finger protein from the  
5 third set.

1 37. The method of claim 36 further comprising identifying subsets of  
2 the first, second and third sets, the subset of the first set comprising zinc finger protein(s)  
3 comprising a finger that specifically binds to the first triplet in the target site from the  
4 first finger position of a zinc finger protein in the database; the subset of the second set  
5 comprising zinc finger protein(s) comprising a finger that specifically binds to the second  
6 triplet in the target site from the second finger position in a zinc finger protein in the  
7 database; the subset of the third set comprising zinc finger protein(s) comprising a finger



8 that specifically binds to the third triplet in the target site from a third finger position in a  
9 zinc finger protein in the database;

10 wherein

11 the outputting step comprising outputting designations and  
12 subdesignations of the subset of the first, second and third sets; and

13 the producing step comprising producing a zinc finger protein comprising  
14 a first finger from the first subset, a second finger from the second subset, and a third  
15 finger from the third subset.

1 38. The method of claim 37, wherein the outputting comprises  
2 outputting the designations and subdesignations of the subsets of the first, second and  
3 third sets, and the first, second and third sets minus their respective subsets.

1 39. The method of claim 38, wherein each of the subsets is a null set.

1 40. The method of claim 35, wherein the target site is provided by user  
2 input.

1 41. The method of claim 35 wherein the target site is provided by the  
2 method of claim 1 or claim 22.

1 42. A method of producing a zinc finger protein comprising:

2 (a) providing a database comprising  
3 designations for a plurality of zinc finger proteins, each  
4 protein comprising at least first, and second fingers,  
5 subdesignations for each of the fingers of each of the zinc  
6 finger proteins; and

7 a corresponding nucleic acid sequence for each zinc finger protein, each  
8 sequence comprising first and second triplets specifically bound by the first and second  
9 fingers respectively, the triplets being arranged in the nucleic acid sequence (3'-5') in the  
10 same respective order as the first and second and fingers are arranged in the zinc finger  
11 protein (N-terminal to C-terminal);

12 (b) providing a target site for design of a zinc finger protein, the  
13 target site comprising contiguous first, and second triplets ordered 3'5' in the target site;

- 14 (c) for the first and second triplet in the target site, identifying first  
15 and second sets of zinc finger protein(s) in the database, the first set comprising zinc  
16 finger protein(s) comprising a finger specifically binding to the first triplet in the target  
17 site, the second set comprising zinc finger protein comprising a finger specifically  
18 binding to the second triplet in the target site;  
19 (d) outputting designations and subdesignations of the zinc finger proteins in the  
20 first, and second sets identified in step (c).

- 1 43 A method of producing a zinc finger protein comprising:  
2 (a) providing a database comprising:  
3 designations for a plurality of zinc finger proteins, each  
4 protein comprising at least first, and second fingers;  
5 subdesignations for each of the fingers of each of the zinc  
6 finger proteins; and  
7 a corresponding nucleic acid sequence for each zinc finger  
8 protein, each sequence comprising first, and second triplets specifically bound by the first  
9 and second fingers respectively, the triplets being arranged in the nucleic acid sequence  
10 (3'-5') in the same respective order as the first and second and fingers are arranged in the  
11 zinc finger protein (N-terminal to C-terminal);  
12 (b) providing a target site for design of a zinc finger protein, the  
13 target site comprising contiguous first, second and third triplets ordered 3'5' in the target  
14 site;  
15 (c) for the first and third triplet in the target site, identifying first  
16 and second sets of zinc finger protein(s) in the database, the first set comprising zinc  
17 finger protein(s) comprising a finger specifically binding to the first triplet in the target  
18 site, the second set comprising zinc finger protein comprising a finger specifically  
19 binding to the third triplet in the target site;  
20 (d) outputting designations and subdesignations of the zinc finger proteins in the  
21 first, and second sets identified in step (c).

- 1 44 A computer program product for selecting a target sequence within  
2 a polynucleotide for targeting by a zinc finger protein, comprising:  
3 (a) code for providing a polynucleotide sequence;

- 4 (b) code for selecting a potential target site within the polynucleotide  
5 sequence; the potential target site comprising first, second and third triplets of bases at  
6 first, second and third positions in the potential target site;
- 7 (c) code for calculating a score for the potential target site from a  
8 combination of subscores for the first, second, and third triplets, the subscores being  
9 obtained from a correspondence regime between triplets and triplet position, wherein each  
10 triplet has first, second and third corresponding positions, and each corresponding triplet  
11 and position has a particular subscore;
- 12 (d) code for repeating steps (b) and (c) at least once on a further potential  
13 target site comprising first, second and third triplets at first, second and third positions of  
14 the further potential target site to determine a further score;
- 15 (e) code for providing output of at least one of the potential target site  
16 with its score
- 17 (f) a computer readable storage medium for holding the codes

1 45. The computer program product of claim 44, further comprising code  
2 for combining a context parameter with a subscore.

- 1 46. A system for selecting a target sequence within a polynucleotide  
2 for targeting by a zinc finger protein, comprising:
- 3 (a) a memory;
- 4 (b) a system bus;
- 5 (c) a processor operatively disposed to:
- 6 (1) provide or receive a polynucleotide sequence;
- 7 (2) select a potential target site within the polynucleotide sequence; the  
8 potential target site comprising first, second and third triplets of bases at first, second and  
9 third positions in the potential target site;
- 10 (3) calculate a score for the potential target site from a combination of  
11 subscores for the first, second, and third triplets, the subscores being obtained from a  
12 correspondence regime between triplets and triplet position, wherein each triplet has first,  
13 second and third corresponding positions, and each corresponding triplet and position has  
14 a particular subscore;

- 15 (4) repeat steps (2) and (3) at least once on a further potential target site  
16 comprising first, second and third triplets at first, second and third positions of the further  
17 potential target site to determine a further score;  
18 (5) provide output of at least one of the potential target site with its score.

1 47. The system of claim 46, wherein the processor is further operatively  
2 disposed to combine a context parameter with a subscore.

1 48. A computer program product for designing a zinc finger protein  
2 comprising:

3 (a) code for providing a database comprising  
4 designations for a plurality of zinc finger proteins, each protein  
5 comprising at least first, second and third fingers ,  
6 subdesignations for each of the three fingers of each of the zinc  
7 finger proteins;  
8 a corresponding nucleic acid sequence for each zinc finger protein,  
9 each sequence comprising at least first, second and third triplets specifically bound by the  
10 at least first, second and third fingers respectively in each zinc finger protein, the first,  
11 second and third triplets being arranged in the nucleic acid sequence (3'-5') in the same  
12 respective order as the first, second and third fingers are arranged in the zinc finger  
13 protein (N-terminus to C-terminus);

14 (b) code for providing a target site for design of a zinc finger protein, the  
15 target site comprising at least first, second and third triplets,

16 (c) for the first, second and third triplet in the target site, code for  
17 identifying first, second and third sets of zinc finger protein(s) in the database, the first set  
18 comprising zinc finger protein(s) comprising a finger specifically binding to the first  
19 triplet in the target site, the second set comprising a finger specifically binding to the  
20 second triplet in the target site, the third set comprising a finger specifically binding to the  
21 third triplet in the target site;

22 (d) code for outputting designations and subdesignations of the zinc finger  
23 proteins in the first, second, and third sets identified in step (c).

24 (e) a compute readable storage medium for holding the codes.

1 49. A system for designing a zinc finger protein comprising:

- 2 (a) a memory;  
3 (b) a system bus;  
4 (c) a processor operatively disposed to:  
5 (1) provide a database comprising  
6 designations for a plurality of zinc finger proteins, each protein comprising  
7 at least first, second and third fingers, subdesignations for each of the three fingers of  
8 each of the zinc finger proteins;  
9 a corresponding nucleic acid sequence for each zinc finger protein, each  
10 sequence comprising at least first, second and third triplets specifically bound by the at  
11 least first, second and third fingers respectively in each zinc finger protein, the first,  
12 second and third triplets being arranged in the nucleic acid sequence (3'-5') in the same  
13 respective order as the first, second and third fingers are arranged in the zinc finger  
14 protein (N-terminus to C-terminus);  
15 (2) provide or be provided with a target site for design of a zinc  
16 finger protein, the target site comprising at least first, second and third triplets,  
17 (3) for the first, second and third triplet in the target site,  
18 identifying first, second and third sets of zinc finger protein(s) in the database, the first set  
19 comprising zinc finger protein(s) comprising a finger specifically binding to the first  
20 triplet in the target site, the second set comprising a finger specifically binding to the  
21 second triplet in the target site, the third set comprising a finger specifically binding to the  
22 third triplet in the target site;  
23 (4) output designations and subdesignations of the zinc finger  
24 proteins in the first, second, and third sets identified in step (3).

1 50. A computer program product for selecting a target site within a  
2 target sequence for targeting by a zinc finger protein comprising:  
3 code for providing a target nucleic acid to be targeted by a zinc finger  
4 protein;  
5 code for outputting a target site within the target nucleic acid comprising  
6 5'NNx aNy bNzc3', wherein

7 wherein each of (x, a), (y, b) and (z, c) is (N, N) or (G, K);

8 at least one of (x, a), (y, b) and (z, c) is (G, K). and

9 N and K are IUPAC-IUB ambiguity codes;

10 and a computer readable storage medium for holding the codes.

1 51. A system for selecting a target site within a target sequence for  
2 targeting by a zinc finger protein comprising:

3 (a) a memory;

4 (b) a system bus;

5 (c) a processor operatively disposed to:

6 provide a target nucleic acid to be targeted by a zinc finger protein;

7 output a target site within the target nucleic acid comprising 5'NNx aNy

8 bNzc3', wherein

9 wherein each of (x, a), (y, b) and (z, c) is (N, N) or (G, K);

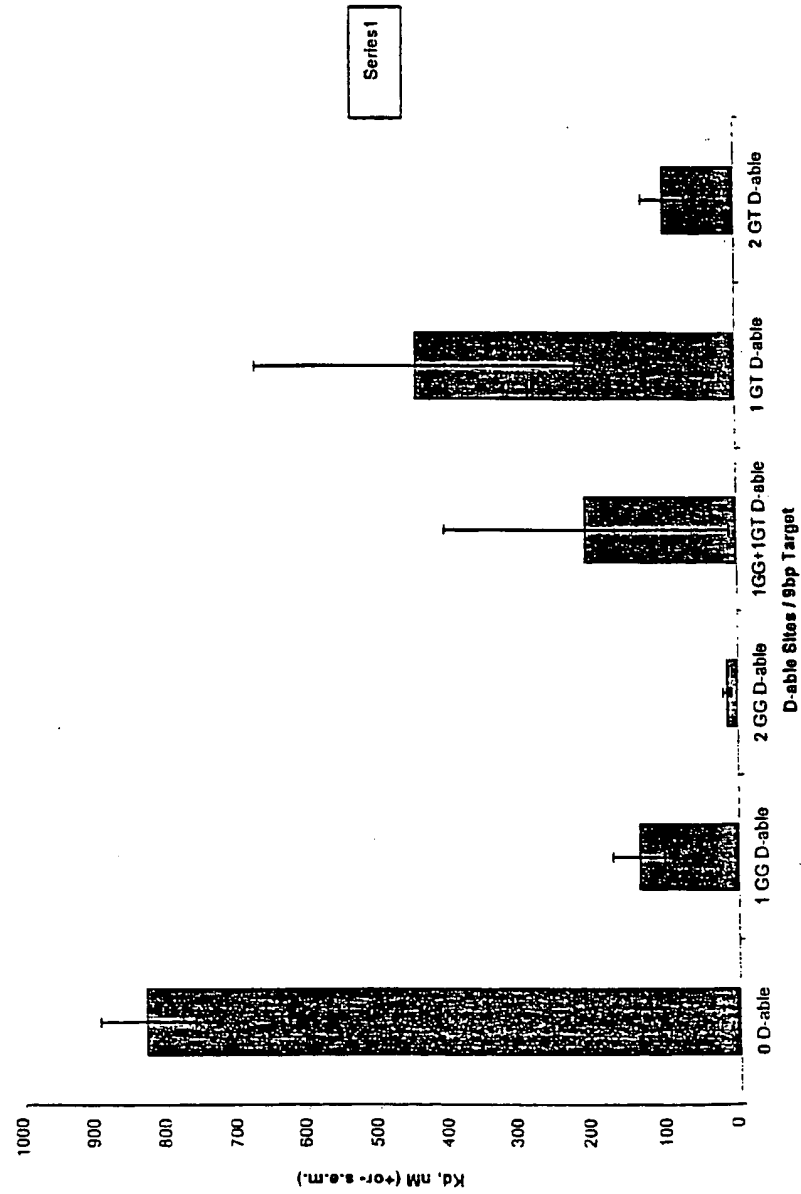
10 at least one of (x, a), (y, b) and (z, c) is (G, K) and

11 N and K are IUPAC-IUB ambiguity codes.

Fig. 1

# Kd of Designed ZFPs vs. D-able Sites / Target:

Affinity is a function of number and type of D-able site



2/10

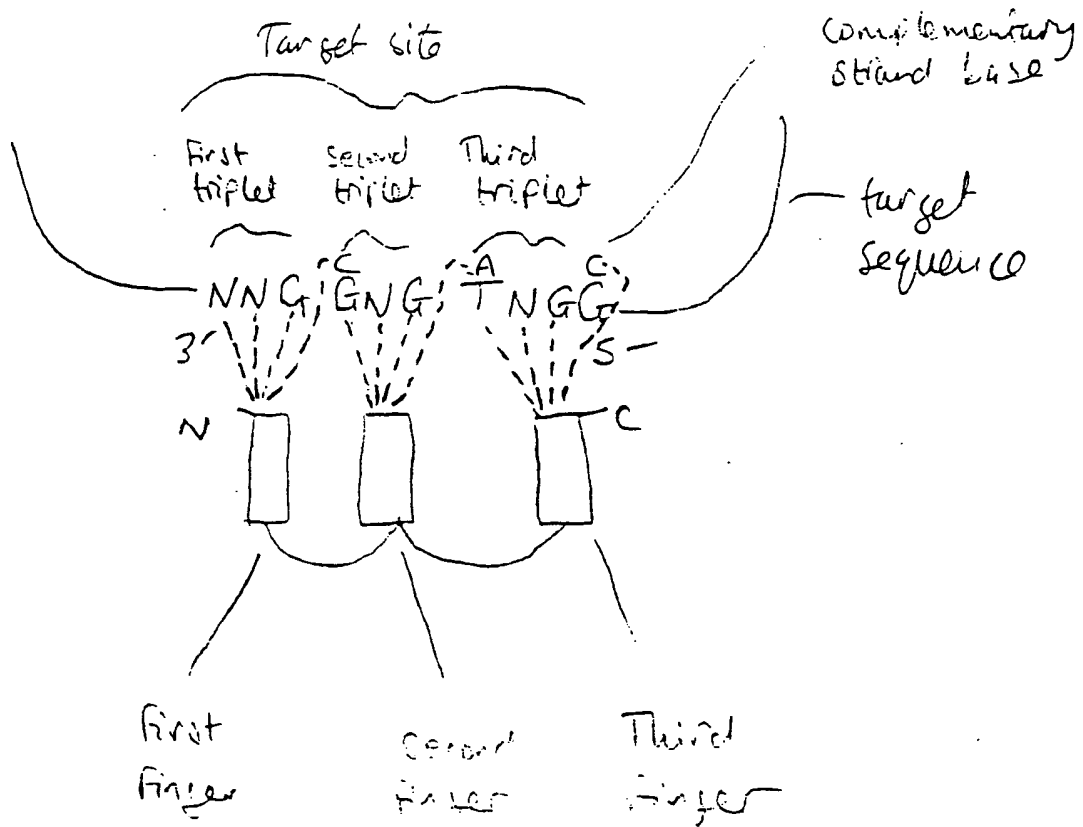
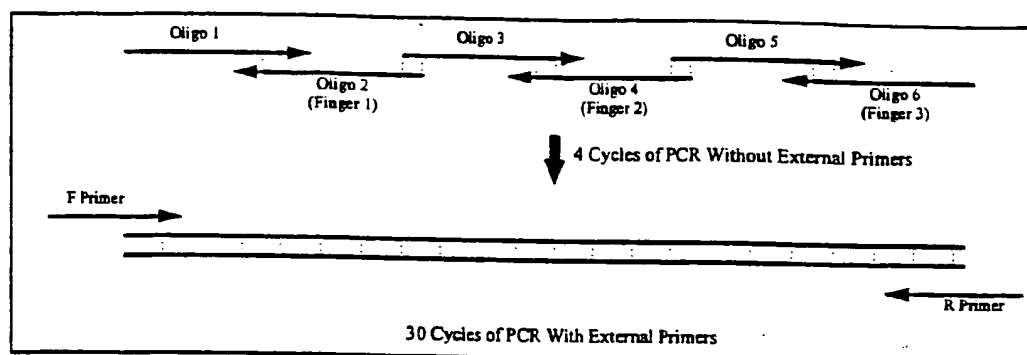


Fig. 2





\*Figure 3. PCR amplification scheme for production of ZFP-encoding synthetic genes.

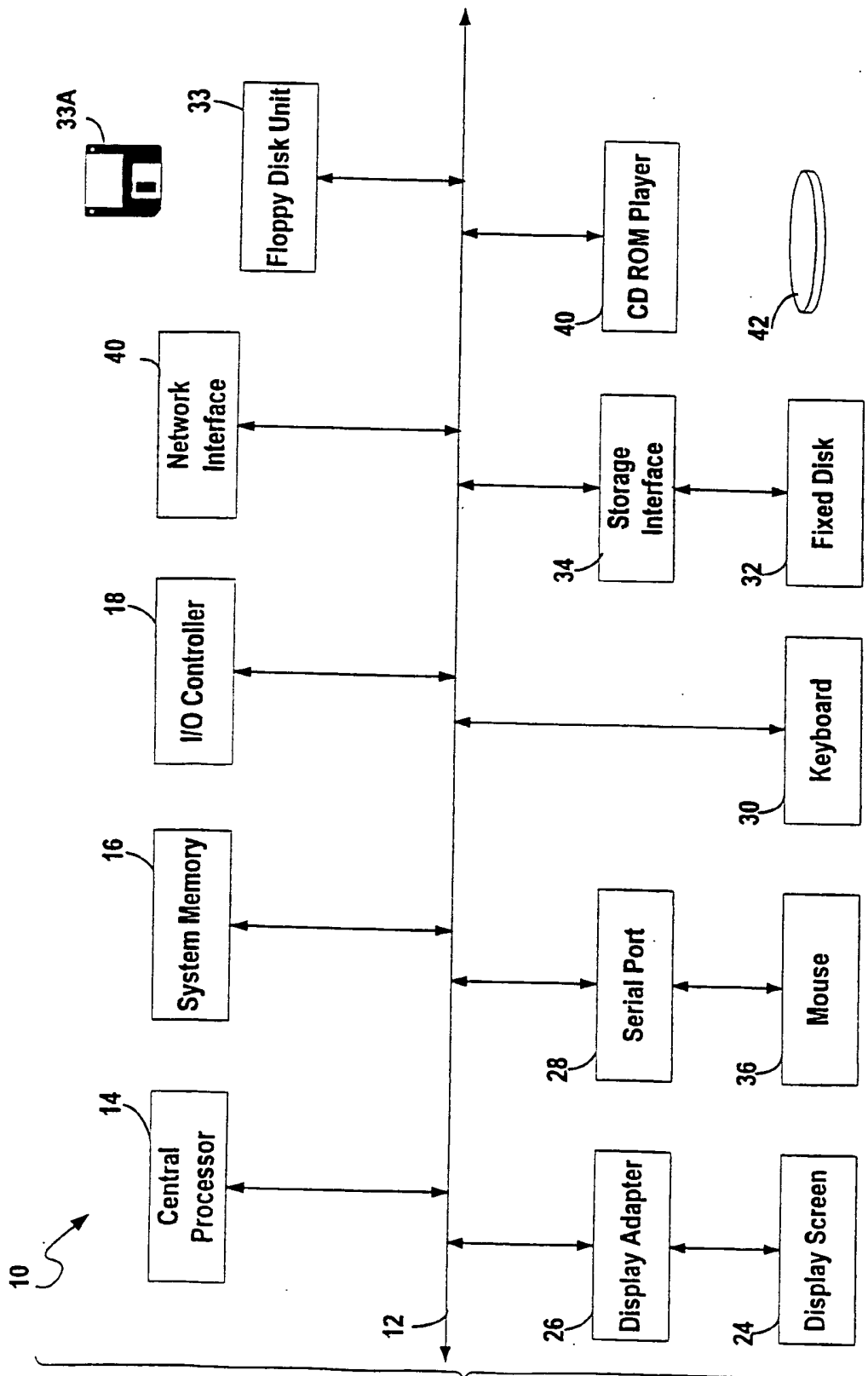


Fig. 4

5/10

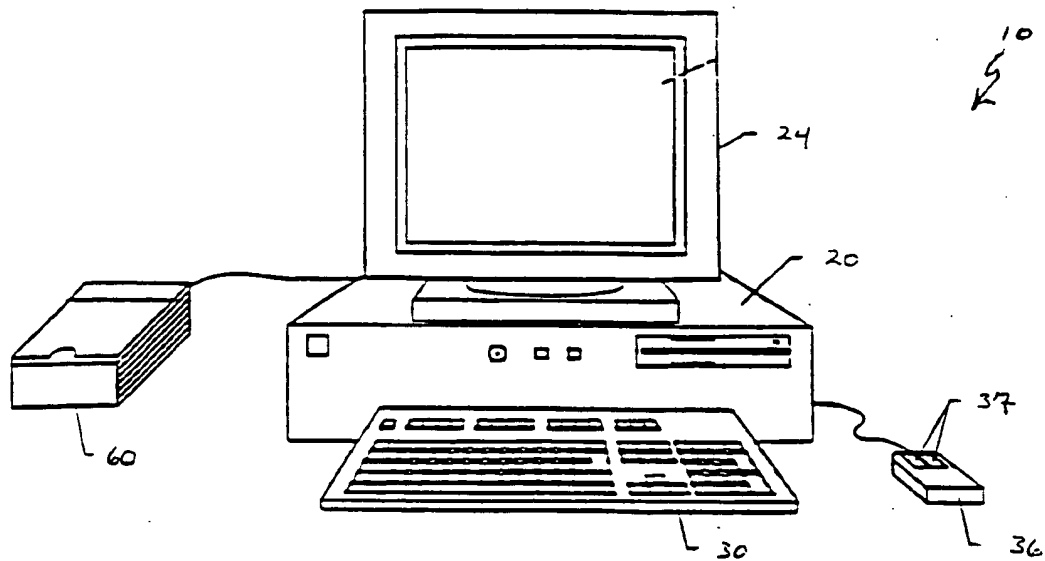


Fig. 5

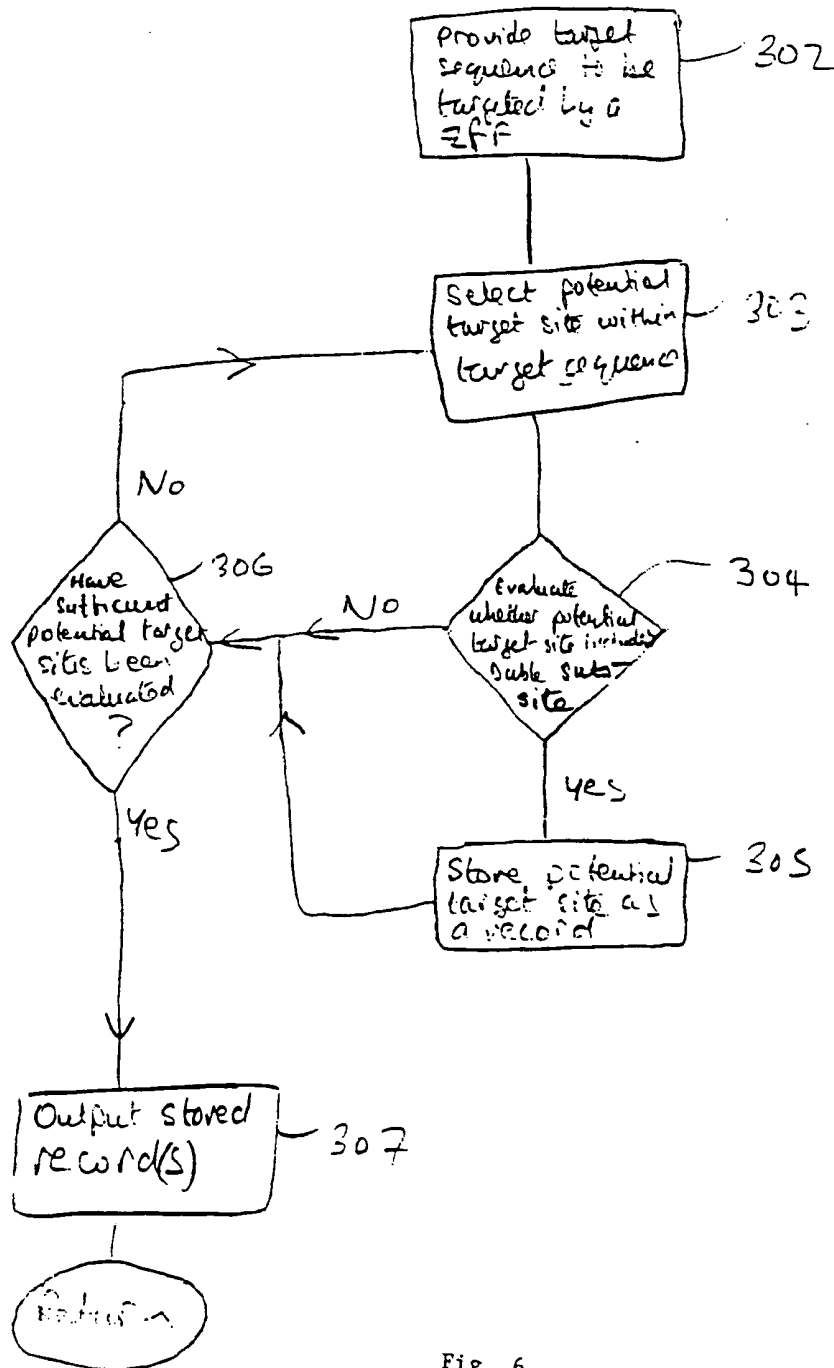


Fig. 6

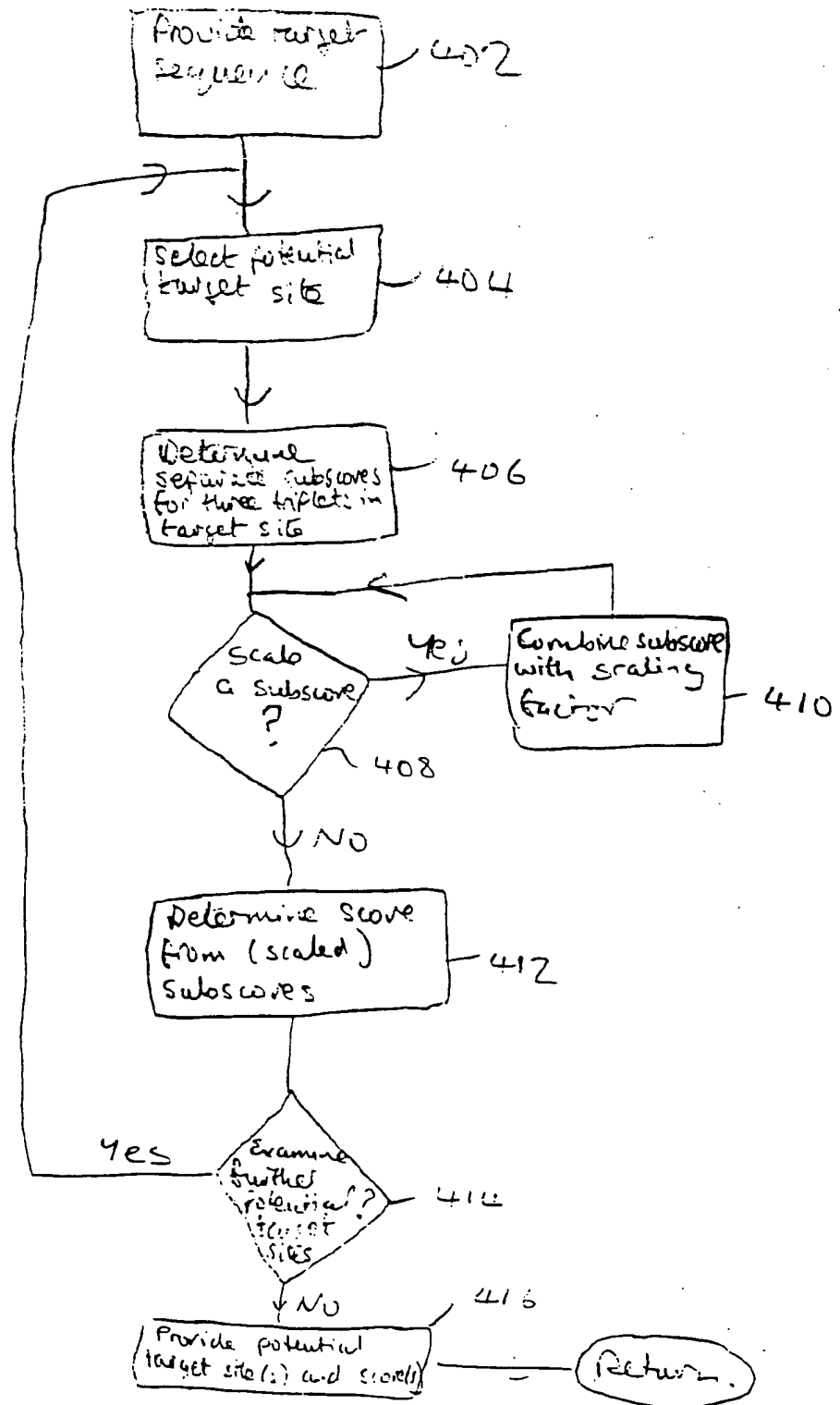


Fig. 7A

8/10

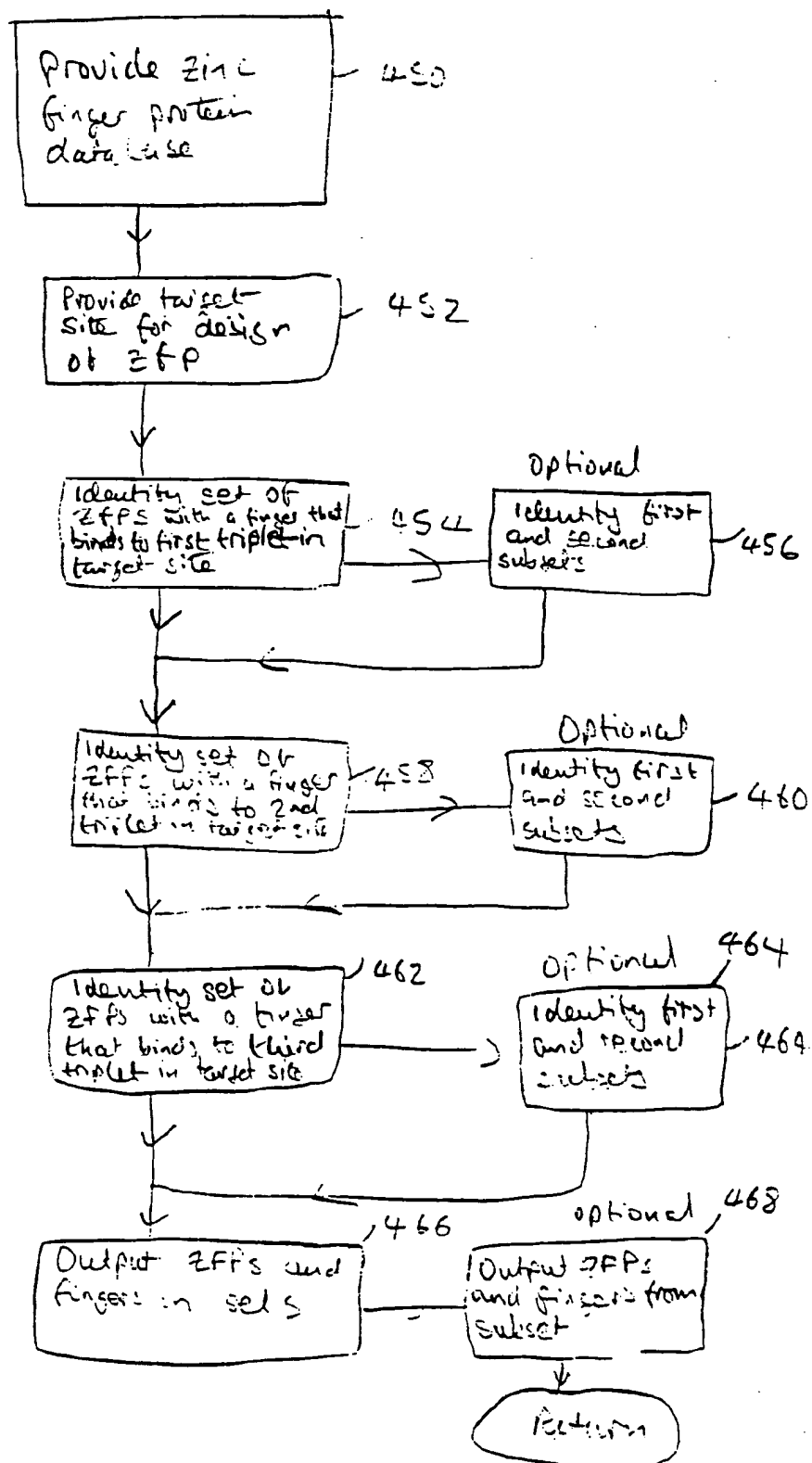


Fig. 7B

9/10

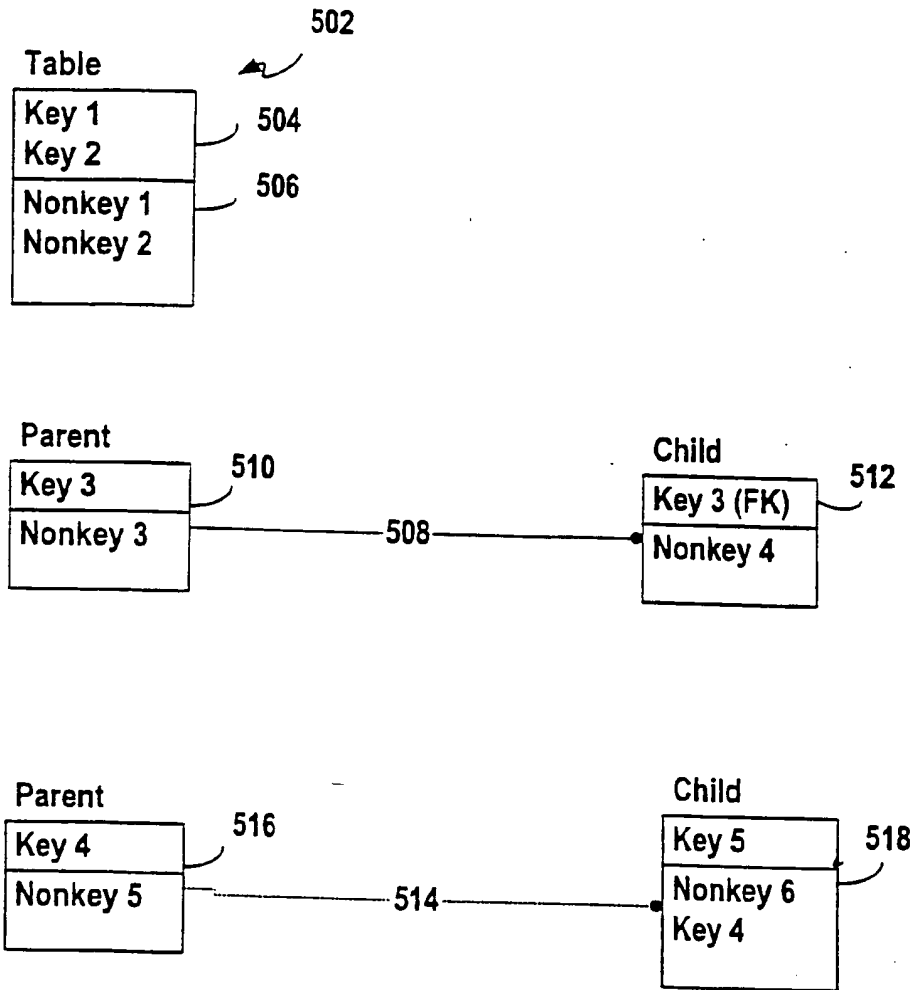


Fig. 8A

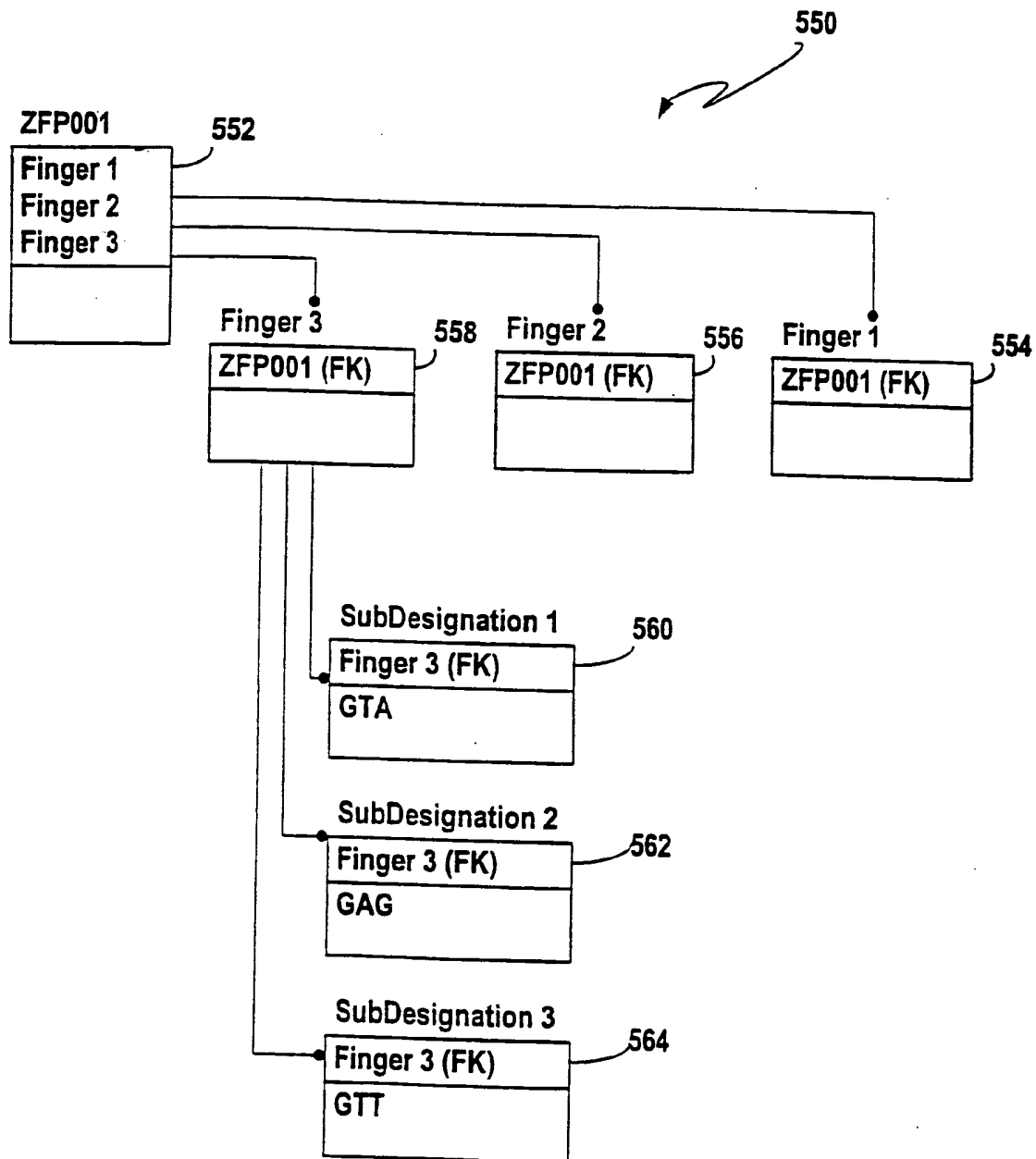


Fig. 8B



## INTERNATIONAL SEARCH REPORT

International application No.  
PCT/US00/00388

<b>A. CLASSIFICATION OF SUBJECT MATTER</b> IPC(7) :C12Q 1/68 US CL :435/6 According to International Patent Classification (IPC) or to both national classification and IPC		
<b>B. FIELDS SEARCHED</b> Minimum documentation searched (classification system followed by classification symbols) U.S. : 435/6  Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched NONE  Electronic data base consulted during the international search (name of data base and, where practicable, search terms used) WEST, STN FILES: MEDLINE, CAPLUS, LIFESCI, EMBASE		
<b>C. DOCUMENTS CONSIDERED TO BE RELEVANT</b>		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A,P	US 6,013,453 A (CHOO et al) 11 January 2000, entire document.	1-51
A	US 5,789,538 A (REBAR et al) 04 August 1998, entire document.	1-51
<input type="checkbox"/> Further documents are listed in the continuation of Box C. <input type="checkbox"/> See patent family annex.		
* "A"	Special categories of cited documents. document defining the general state of the art which is not considered to be of particular relevance	"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention.
"B"	earlier document published on or after the international filing date	"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"O"	document referring to an oral disclosure, use, exhibition or other means	"Z" document member of the same patent family
"P"	document published prior to the international filing date but later than the priority date claimed	
Date of the actual completion of the international search 20 MARCH 2000		Date of mailing of the international search report 18 APR 2000
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230		Authorized officer JOYCE BRIDGERS PARALEGAL SPECIALIST JANELL TAYLOR CHEMICAL MATRIX Telephone No. (703) 308-0196

**THIS PAGE BLANK (USPTO)**